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Exploration of Helminth-derived Immunoregulatory Molecules as Options for Therapeutic Intervention in Allograft Rejection and Autoimmune Disease

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Declaration

I declare that this thesis has been composed by myself, describes my own work and has not been submitted in any other application for a higher degree.

Sections of the Introduction and the Materials and Methods chapters have been previously published (as first author); the published articles are included in appendix A.

Additional contributions are acknowledged in the next section, but particular mention is also made here of Figure 4-8, which was the result of experiments performed jointly, but very much led by Dr Danielle Smyth. Also, macromolecular crowding assays featuring in Figures 5-14 and 5-15 were performed by Dr Hannah Woodcock at University College London, as the result of a collaboration that I established for this purpose.

Chris Johnston
July 2015

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A considerable number of people have been generous with their time and expertise in assisting with this project – some are mentioned here and I apologise to the many who are not.

First, in tackling this beast, I could not have asked for better supervision. The Wellcome Trust's ECAT scheme managed to draw together Prof Rick Maizels, Prof Steve Anderton and Prof Steve Wigmore in a collaboration that might not otherwise have happened. This has worked out extremely well and I'm grateful to all three for the time and effort that has been dedicated. As primary supervisor, Rick has undoubtedly suffered the most, but without it ever showing. Last-minute emails and deadline crises still receive good-humoured responses.

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A sample of individual contributions includes:
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Abstract

Solid organ transplantation is the gold standard treatment for a variety of conditions that result in organ failure. However, despite considerable advances in clinical transplantation in recent decades, the almost ubiquitous requirement of life-long immunosuppression of transplant recipients persists and is complicated by graft loss to rejection in the long term and multiple serious adverse effects that are frequently life limiting.

Helminths currently infect more than one quarter of the world's population and it is now well established that their success as parasites is the result of active immunomodulation of the host immune response. Whilst this primarily secures on-going survival of the parasites, in some cases helminth-induced immunomodulation can be beneficial to the infected host and is not associated with the adverse sequelae of pharmacological immunosuppression. An emerging body of evidence suggests that harmful immune responses to alloantigens can be suppressed by helminths, but little mechanistic data exists and the active immunomodulators involved have remained hitherto unidentified.

The hypothesis behind this thesis is that the model intestinal nematode, *Heligmosomoides polygyrus*, produces immunomodulatory molecules that can suppress responses to allo- and auto-antigens in animal models of transplantation and autoimmunity, and that some of these molecules could potentially be exploited as novel therapeutic agents.

Full-thickness skin grafting was performed between fully-allogeneic mouse strains (BALB/c to C57BL/6). Recipient mice infected with H. polygyrus immediately prior to transplantation showed significantly prolonged allograft survival. Likewise, protection from allograft rejection could be replicated in recipient mice in which *H. polygyrus* excretory-secretory products (HES) (isolated from culture of adult worms) were delivered by continuous infusion via surgically implanted osmotic minipumps. A number of potential mechanisms underlying allograft protection were identified

including induction of $CD4^+CD25^+Foxp3^+$ regulatory T cells (Treg) and suppression of Th1 and Th17 effector $CD4^+$ T cell phenotypes.

H. polygyrus and HES were further shown to ameliorate disease in murine (pMOG) experimental autoimmune encephalomyelitis and colitis induced by T cell transfer. In addition to expansion of Treg, H. polygyrus-mediated protection against EAE was found to be almost completely lost in IL-4 receptor deficient mice, indicating a protective role of Th2 immune responses in this context.

Finally, the mechanisms of action of the newly-identified TGF-β mimic, TGM, contained within HES were investigated. Despite bearing no sequence homology or structural resemblance to TGF-β, TGM was shown to act through the TGF-β receptor complex to induce Treg in human and mouse CD4+ T cells in vitro and to suppress murine allogeneic skin graft rejection in vivo. TGM may represent the origin of a safe, effective and long-overdue novel alternative to current immunosuppression therapy.

Abbreviations

AF700	Alexa-Fluor 700
APC	Antigen presenting cell *or* allophycocyanin
BSA	Bovine serum albumin
CBA	Cytokine Bead Array
CD	Cluster of differentiation
CFA	Complete Freund's adjuvant
CPM	Counts per minute
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DO11.10	A transgenic T cell receptor specific for ovalbumin
EAE	Experimental autoimmune encephalomyelitis
ELISA	Enzyme linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FCS	Foetal calf serum
FITC	Fluoroisothiocyanate
Foxp3	Forkhead box P3
FSC	Forward scatter
GATA3	GATA binding protein 3

H&E	Haematoxylin and eosin
HBSS	Hanks' Balanced Salt Solution
HES	*Heligmosomoides polygyrus* excretory/excretory products
HMGB1	High mobility group box 1
HS	Highly significant
IP	Intraperitoneal
IRI	Ischaemia reperfusion injury
iTreg	Induced (*In vitro*) regulatory T cell
IV	Intravenous
IFN	Interferon
Ig	Immunoglobulin
IL-X	Interleukin-X
IL-XR	Interleukin-X receptor
KJ1.26	A monoclonal antibody specific for the DO11.10 TCR
LN	Lymph node
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MACS	Magnetically activated cell sorting
MHC	Major histocompatibility complex
min	Minutes

mRNA	Messenger ribonucleic acid
OD	Optical density
PAGE	Poly-acrylamide gel electrophoresis
PAMPs	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PD-1	Programmed Cell Death Protein 1 (CD279)
PE	Phycoerythrin
PerCP	Peridinin-chlorophyll-protein complex
PMA	Phorbol 12-myristate 13-acetate
PNPP	p-nitrophenyl phosphate
PRRs	Pattern recognition receptors
pTreg	Regulatory T cell induced outside the thymus (*in vivo*)
RA	Retinoic acid
RNA	Ribonucleic acid
RORγt	Retinoic acid-related orphan receptor γt
SC	Subcutaneous
SEA	Soluble egg antigen from *Schistosoma mansoni*
SSC	Side scatter
ST2	Interleukin 1 receptor-related protein

Tbet	T box expressed in T cells
TCR	T cell receptor
TGF-β	Transforming growth factor beta
TGM	Transforming growth factor beta mimic
Thx	T helper x
TLR	Toll-like receptor
TNF	Tumour necrosis factor
tTreg	Regulatory T cell induced in the thymus
WT	Wild-type

1 Introduction

The necessity for life-long immunosuppression following organ transplantation is associated with multiple serious adverse consequences, such that the life expectancy of transplant recipients is frequently curtailed as a direct result of the medication prescribed for them. This is a predicament that has changed little over the last thirty years and the need for novel therapeutic options is clear. In this introduction, the current state of clinical organ transplantation will be explored, followed by an overview of helminth parasites' immunomodulatory capabilities in relation to autoimmunity and transplantation, and conclude with an outline the remaining questions this thesis aims to answer.

1.1 Solid Organ Transplantation

1.1.1 The Role for Transplantation

Organ transplantation is now widely accepted as the gold standard treatment for a vast array of diseases that culminate in organ failure[1]. In comparison to dialysis, kidney transplantation has been shown to be beneficial in terms of improved survival (mortality 68% lower than that of patients on the transplant waiting list[2]), quality of life and cost effectiveness. The comparative health and economic benefits of transplantation are seen in all groups of potential recipients, including those with advanced age or co-morbidity[3]. However, despite consistently excellent short-term outcomes of organ transplantation, results in the longer term have been disappointing, largely as a result of chronic allograft rejection and toxicity from currently-employed immunosuppressive agents (Figure 1-1)[4]. Results from a small number of trials aiming to minimise immunosuppression following kidney transplantation were also not encouraging and showed that most patients who did develop an episode of acute rejection on minimization protocols ultimately lost their grafts[5,6].

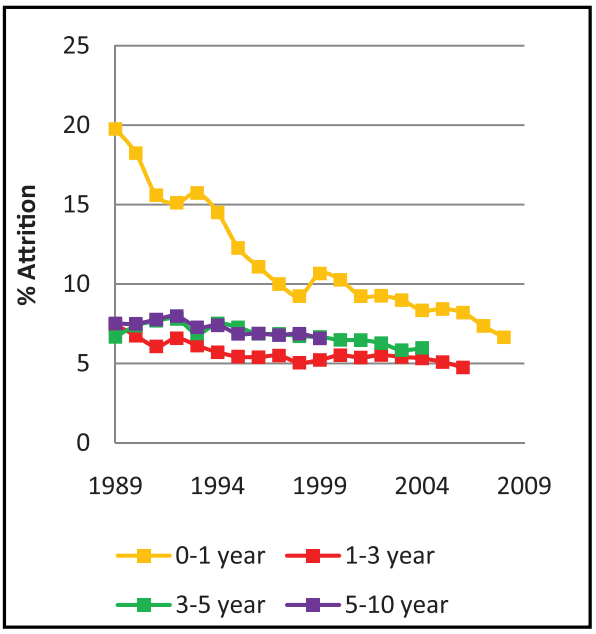


Figure 1-1 Cumulative graft failure yearly attrition rates of first kidney transplant. Reproduced from Lamb *et al*[7].

1.1.2 Current Clinical Practice and Challenges

Until the 1990s, transplantation from cadaveric donors was performed almost exclusively following brain death, known as 'Donation after Brain Death (DBD)'[8]. The available pool of 'ideal' donors following brain death has inexorably decreased however, largely as a result of reductions in fatal road traffic trauma and improvements in blood pressure control (reducing the incidence of fatal cerebral haemorrhage)[9]. With the requirement for donated organs continuing to increase, necessity drove a number of strategies to increase the available pool of donor organs. Some of these strategies present additional immunological challenges, such as donation from HLA- or ABO-incompatible donors, donation after circulatory death (with the potential for allograft damage from longer periods of warm ischaemia) and acceptance of suboptimal 'extended criteria donors'[10,11]. However, living donor donation has also increased (figure 1-2) and presents novel therapeutic opportunities in advance of the transplant taking place.

Whilst the number of organs donated following brain death (DBD) has remained relatively static over the five years preceding 2013 (figure 1.2), both the age and body mass index of donors within this group has increased considerably[12]. In the context of kidney transplantation, organs from elderly donors have been associated with poorer graft and recipient survival rates[13], but this still compares favourably to the mortality associated with remaining on maintenance dialysis[14,15].

Donation after circulatory death (DCD) engenders a number of technical, logistical and legal difficulties. Prolonged organ ischaemia is of foremost concern, particularly as the law in most countries requires a specified 'no touch' period between the diagnosis of death and commencement of the organ retrieval operation (e.g. five minutes)[8]. Increased utilisation of DCD donors in the U.K. has played fundamental role in increasing the number of transplant operations performed (figure 1.2), to the extent of marginally reducing the transplant recipient waiting list in 2011[12].

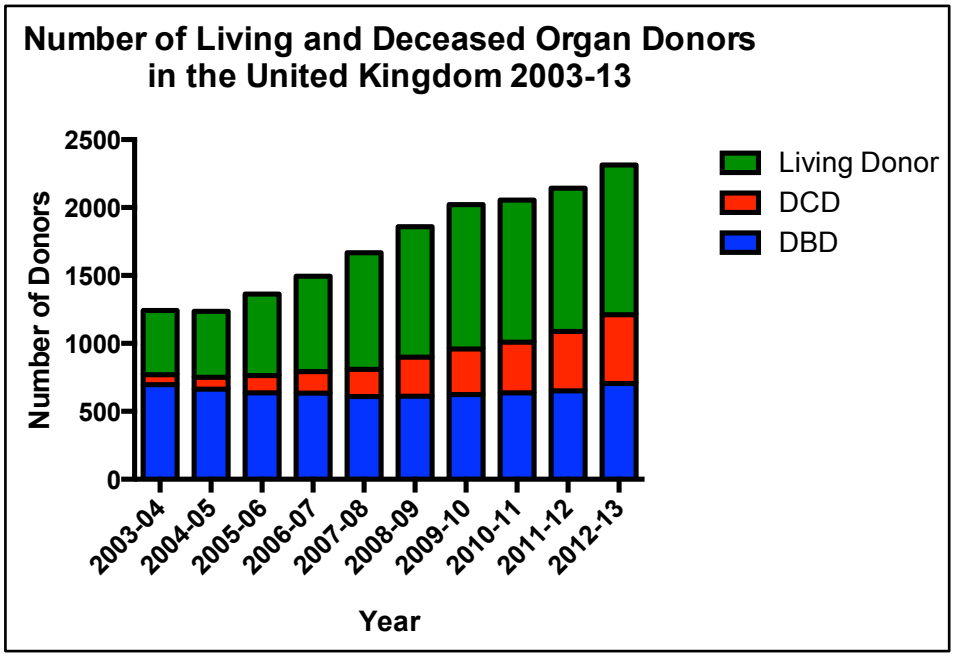


Figure 1-2 Transplanted Organs in the United Kingdom according to donor type. Source: NHSBT. (DCD: Donation after Circulatory Death; DBD: Donation after Brain Death)

Until recently in the United States, up to 30% of potentially suitable living donors were withdrawn from the transplantation process following detection of circulating donor-specific ABO or HLA antibodies on initial screening[16]. At the same time, cultural unacceptability of transplantation from cadaveric donors in many Asian countries drove the development of aggressive approaches to overcoming immunological barriers to living donor donation. Successful ABO-incompatible donation programmes started in Japan with a protocol including pre-transplant splenectomy[17], but have been refined over time with consecutively less invasive protocols showing similarly impressive outcomes in the short term (from the limited follow up data available)[18,19]. One major drawback of ABO-incompatible transplantation is cost: additional interventions including pre-transplant plasmapheresis, anti-CD20 conditioning and frequent antibody titer monitoring results in the transplant procedure costing up to 72% more than the ABO-compatible equivalent[20]. As a result, whilst the number of 'incompatible' transplants has increased considerably in recent years (figure 1-3), further expansion will likely be limited on the grounds of financial feasibility and availability of the required expertise.

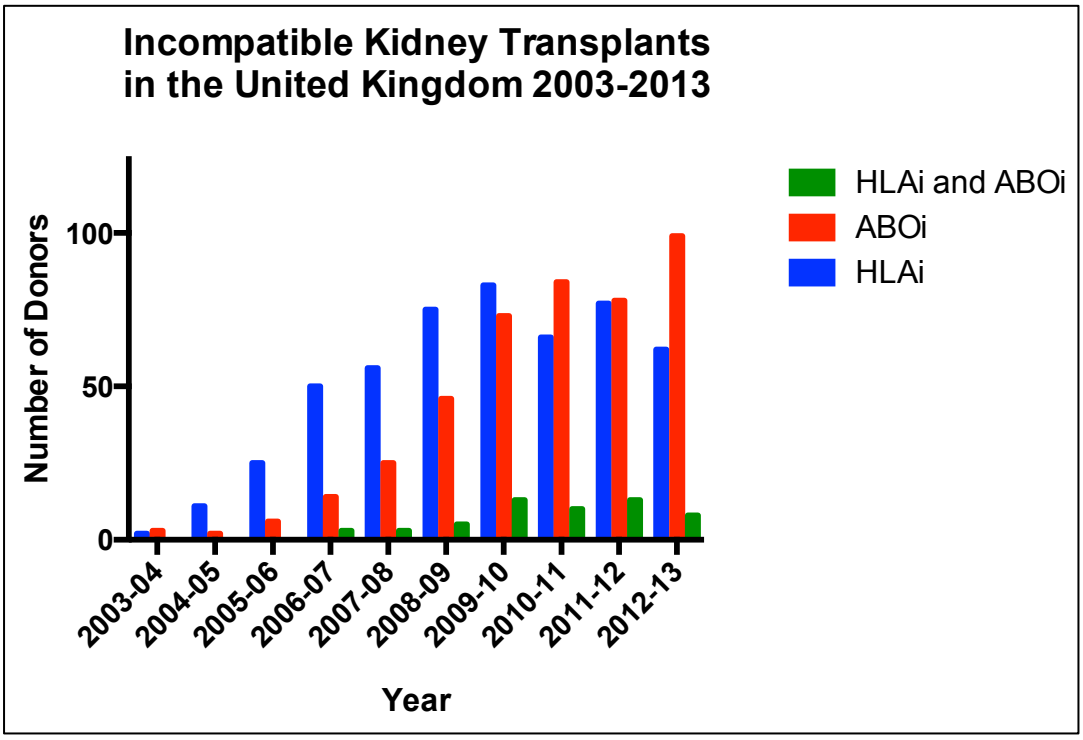


Figure 1-3 Source: NHSBT. HLAi: Human Leukocyte Antigen incompatible; ABOi: ABO incompatible.

1.1.3 Fundamentals of Allograft Rejection

1.1.3.1 Innate Immunity

The physical process of surgically explanting an organ and then subjecting it to a period of ischaemia prior to reperfusion in another patient inevitably leads to a degree of organ damage, resulting in the stimulation of an innate immune response[21]. The innate response occurs irrespective of the degree of allogeneic mismatch and, by inviting an alloantigen-specific adaptive immune response (and subsequently collaborating with it), innate immunity plays a fundamental role in determining graft outcome[22]. Ischaemia-reperfusion injury (IRI) arises when reperfusion of hypoxic tissue generates oxygen free radicals, resulting in cell damage and an inflammatory response that causes tissue injury in excess of that produced by ischaemia alone[23]. IRI is one of the main contributors to allograft damage and likely contributes to both

the comparatively favourable outcomes of living donor transplantation[24] and the positive results demonstrated by *ex-vivo* perfusion of explanted allografts compared to cold preservation[25].

Innate immune cells express pattern recognition receptors that enable them to recognise tissue injury through detection of characteristic endogenous protein signatures of damaged cells, known as damage-associated molecular patterns (DAMPs)[26]. Toll-like receptors (TLRs) are a ubiquitous group of highly conserved transmembrane pattern recognition receptors that recognise DAMPs from damaged cells and are upregulated during IRI[27]. DAMPs are expressed in pathological conditions such as hypoxia or oxidative stress (e.g. following donor brain death) and consist of a varied group of molecules including heat shock proteins, adenosine triphosphate (ATP), purines and the DNA-binding protein, high mobility group box 1 (HMGB1)[28]. Ligation of TLRs by DAMPs (except TLR3) initiates an intracellular signalling cascade through the adapter protein, myeloid differentiation factor 88 (MyD88). This leads to the release of inflammatory cytokines (IL-1, IL-6 and TNF) and chemokines, initiating a series of events that includes production of acute phase proteins and increased vascular permeability, allowing donor APCs to migrate to recipient draining lymphoid tissue and recipient inflammatory leucocytes to be specifically recruited into the allograft[26]. Genetically deficient mouse strains that are protected from injury in murine models of IRI include $MyD88^{-/-}$ mice, nude mice (lacking T cells)[29] and $Stat4^{-/-}$ mice (lacking Th1 cells)[30]; demonstrating the critical role of TLR signalling and subsequent generation of an adaptive Th1 response.

The complement system consists of a highly conserved set of proteins that allows for rapid escalation of a non-specific innate immune response to cell damage or pathogens[31]. Complement cascades all converge to mediate enzymatic cleavage of the third complement component, C3, into C3a and C3b. This stimulates multiple further enzymatic cascades resulting in the formation of a lytic 'membrane attack complex' (MAC) and multiple other soluble complement proteins that act as opsonins, chemoattractants and activating ligands for innate immune cells such as macrophages and neutrophils[32]. Further to this, C3a and C5a promote Th1 differentiation of naïve $CD4^{+}$ T cells, thereby inducing an adaptive immune response

that will contribute to allograft rejection[33]. Activation of the complement system is seen predominantly in IRI and episodes of acute rejection, and is associated with poor allograft outcomes in the medium term[27]. Interestingly, complement activation appears to be very much dependent on localised production of C3. Transplantation of a $C3^{-/-}$ kidney into a WT recipient mouse results in significant protection from IRI compared to a WT donor organ[34]. This suggests that an unusual situation arises whereby a transplanted allograft can become complicit in its own destruction and therefore intervention targeted at the donor organ may be a viable therapeutic prospect[35].

1.1.3.2 Alloantigen recognition by T cells

The critical role of T cells in mediating allograft rejection is long-established: mice lacking T cells are unable to reject fully-allogeneic grafts[36] and pharmacological T cell depletion is an effective therapeutic option for terminating episodes of acute rejection in the clinical setting[37]. Initiation of the adaptive immune response occurs mainly via two distinct pathways: direct recognition by T cells of allogeneic MHC molecules on the surface of transplanted donor cells (direct pathway)[38] and via recognition of donor antigens that have been processed and presented on the MHC molecules of the transplant recipient's APCs (indirect pathway)[39]. A third mechanism, semi-direct allorecognition, has also been described, whereby intact donor MHC-peptide complexes are presented on the surface of recipient dendritic cells[40], a situation arising as a result of cell-cell contact[41], or the transfer of intact MHC-peptide complexes via exosomes arising from the donor cells[42]. The existence of a semi-direct pathway presents a resolving solution as to how a single recipient APC can stimulate both $CD8^+$ T cells (via 'semi-direct' recognition of an intact donor MHC-peptide complex) and $CD4^+$ T cells (via 'indirect' recognition of processed donor peptides, presented on recipient MHC complexes)[43,44].

Classically, the direct pathway of allorecognition has been deemed responsible for acute rejection, whilst the indirect pathway of antigen presentation is responsible for chronic rejection. This is intuitively plausible: 'passenger' donor APCs in the

transplanted allograft are immediately available for allorecognition, but will disappear over time, whereas the indirect pathway requires some time for processing of allogeneic peptides, but may continue for the life of the graft. More recently, this strict dichotomy has been questioned and the indirect pathway has been shown to play a role in acute rejection as well[45].

In addition to recognition of allogeneic peptides, T cell activation requires a second signal in the form of co-stimulation. An extensive array of co-stimulatory molecules exists[46], of which the most well-described is CD28. CD28 is constitutively expressed by T cells and ligated by CD80 and CD86 on the surface of APCs. Expression of CD80 is upregulated rapidly during an innate immune response, whist CD86 appears later with sustained APC activation. Ligation of CD28 leads to IL-2 production, T cell proliferation and a lower threshold for T cell activation[47]. Cytotoxic T lymphocyte antigen-4 (CTLA-4 / CD152) is a co-inhibitory molecule that also binds to CD80 and CD86, but with greater affinity and, in contrast to CD28, inhibits T cell activation through transendocytosis; a process that allows CTLA-4-expressing cells to engage, remove and subsequently degrade CD80 and CD86 from target cells[48,49]. More recently, additional co-inhibitory molecules have been discovered, including programmed cell death protein 1 (PD-1 / CD279). PD-1 is widely expressed on activated immune cells (including T cells, B cells and macrophages[50]); it recognises the ligands PDL-1 and PDL-2 on the surface of interacting cells and plays an important role in the maintenance of self-tolerance ($PD\text{-}1^{-/-}$ mice show increased susceptibility to systemic autoimmunity)[51]. Ligation of PD-1 dampens T cell activation, proliferation and production of inflammatory cytokines[52]. In transplantation, PD-1 signalling appears to play an important role both in directly suppressing alloreactive T cell responses[53] and in promoting differentiation of naïve CD4+ T cells into Treg[54].

1.1.3.3 B Cells

The capability of B cells to mediate graft destruction is most dramatically illustrated by clinical episodes of hyperacute rejection, where an allograft is transplanted into a

recipient with pre-formed antibodies, resulting in rapid activation of complement and clotting cascades, widespread thrombosis and graft infarction, often within minutes of reperfusion. This extremely rare scenario has historically arisen following transplantation of ABO-incompatible[55] or HLA-incompatible allografts[56].

B cells play multiple roles in the alloimmune response. First, they can act as APCs, presenting alloantigens and co-stimulation to T cells in either a pro-inflammatory or regulatory fashion[57]. In presenting alloantigens to T cells, cell clusters are formed, where activated T cells can produce cytokines to act in a paracrine fashion and influence adjacent B cell activation, differentiation and antibody production[35]. In this way, immature B cells develop into memory B cells or plasma cells, producing alloantigen-specific antibodies[58]. Antibody deposition within the allograft leads to activation of the classical complement cascade, which can effect graft destruction directly, through formation of the MAC and indirectly through recruitment of neutrophils and macrophages[59].

The risk of antibody-mediated rejection is clearly increased by sensitising events such as previous transplants, blood transfusions or pregnancies; but previous infections can also play a role. Memory B cells (and T cells) generate an amplified and more rapid response upon re-exposure to their cognate antigen. Over time and the course of repeated infections, an ever-expanding repertoire of memory B cells accumulates and, as a result of antigen cross-reactivity, this can underlie an increased risk of heterologous antibodies showing affinity for donor antigens in the elderly transplant recipient[60].

1.1.3.4 Th1 $CD4^+$ T Cells

Sub-classification of $CD4^+$ helper T cells according to their transcription factor and cytokine profile began with the Th1/Th2 paradigm in 1986[61]. Since then, it has become clear that numerous subtypes exist, into which naïve $CD4^+$ T cells can differentiate, and that these types are generally mutually exclusive. The mature phenotype of an activated $CD4^+$ T cell is determined by a number of factors

including antigen concentration, co-stimulation and the surrounding cytokine environment[62,63].

Th1 lymphocytes develop upon exposure to antigen in the presence of IL-2 and IL-12; they are identified by the transcription factor 'T box expressed in T cells' (Tbet, *Tbx21*) and production of IFN-γ and are functionally involved in the effector T cell response during intracellular pathogen infection, organ-specific autoimmunity and allograft rejection[64]. Numerous mechanisms of Th1-mediated allograft rejection have been described. First, Th1 lymphocytes produce IL-2 and can therefore promote the proliferation of $CD8^+$ cytotoxic T cells. As $CD8^+$ T cells produce IFN-γ (which promotes Th1 differentiation), a reciprocal positive feedback arrangement can be established, thereby amplifying allogeneic effector T cells and rapidly contributing to rejection[64]. Secondly, release of Th1 cytokines (including IL-1, IFN-γ and TNF) augments cytotoxicity by recruiting macrophages to release additional non-specific mediators such as reactive oxygen species and nitric oxide. Thirdly, Th1 lymphocytes are capable of directly killing target cells through expression of Fas ligand (FasL) which, when recognised by Fas receptors (CD95) on the surface of target cells, induces apoptosis[65]. Finally, Th1 cytokines also activate B cells to produce alloantigen-specific antibodies, augmenting the rejection process, as previously discussed. In addition to animal models suggesting a Th1-mediated process of rejection, T cells isolated from rejecting kidneys in human transplant recipients show elevated expression of Tbet and FasL compared to isolates from allografts not undergoing rejection[66]. Upon *ex-vivo* restimulation, $CD4^+$ T cells from rejecting organs also secrete IFN-γ (and not IL-4 or IL-5)[67].

1.1.3.5 Th2 $CD4^+$ T Cells

As one of the primary roles of Th1 lymphocytes is to mount a powerful immune response against rapidly proliferating pathogens, the risk of collateral damage (harm to self tissues or structures) is an acceptable evolutionary tradeoff. By contrast, Th2 responses are often induced by helminth infections; organisms that do not complete their life cycle (and therefore proliferate) inside their hosts, but do penetrate through

tissues and mucosal barriers with the potential for considerable harm. In this circumstance, therefore, a different imperative exists: control of parasite numbers is not an immediate priority, but repair of injury and prevention of further collateral damage very much is[68]. Accordingly, Th2 immune responses are predominantly involved in promoting wound repair and limiting other inflammatory responses (discussed in more detail later).

Th2 lymphocytes are characterised by the transcription factor GATA-3 and a cytokine profile dominated by IL-4, IL-5 and IL-13. Th2 cytokines inhibit Th1 responses and it has therefore long been suggested that a Th2 response may be capable of inhibiting or preventing allograft rejection[69]. Indeed, administration of exogenous IL-4 has previously been shown to prolong cardiac allograft survival in rats by 43% and was suggested as a possible therapeutic strategy[70]. However, other studies suggest that Th2 responses are capable of mediating rejection of murine islet[70] and cardiac allografts, possibly as a result of IL-5-driven eosinophil recruitment[70]. Subsequently, Illigens *et al*[71] demonstrated that a Th2 response abrogated $CD8^+$ T cell allograft infiltration, markedly delaying rejection of single class I MHC-mismatched skin grafts, but did also induce *de novo* chronic rejection in cardiac transplants of the same allogeneic combination. Additionally, one clinical study of 102 kidney transplant recipients has shown a significant correlation between genetic polymorphisms associated with low IL-4 production and protection from chronic allograft rejection[72]. In total, the available evidence therefore suggests that (1) Th2 responses may protect allografts from acute rejection by suppressing Th1 and, particularly, $CD8^+$ effector mechanisms; (2) acute rejection may still occur indirectly, as a result of Th2-mediated eosinophil recruitment and (3) in the longer term, Th2 activity may contribute to chronic allograft damage secondary to fibrosis.

1.1.3.6 Th17 $CD4^+$ T Cells

Since 2005, the Th1/Th2 paradigm has been expanded to encompass numerous $CD4^+$ effector T cell subtypes including Th17 cells[73], identified by the transcription factor RORγt and secretion of cytokines including IL-17, IL-21 and IL-22. The discovery

of Th17 lymphocytes resolved a number of anomalous experimental findings. Foremost amongst these, Miura *et al* showed that fully-allogenic cardiac allografts were subject to accelerated rejection in IFN-$\gamma^{-/-}$ recipient mice compared to WT recipients[74]. In these experiments, the pathological processes leading to rejection also appeared to be different: WT recipients showed a characteristic mononuclear cellular infiltration of the allograft comprised mainly of $CD8^+$ T cells, whilst IFN-$\gamma^{-/-}$ recipient allografts had very few infiltrating lymphocytes, but did show an intense neutrophil infiltration with extensive surrounding parenchymal necrosis of the graft. Depletion of neutrophils in these animals successfully prevented accelerated rejection, indicating a causative role[74]. Subsequently, Yuan *et al* demonstrated accelerated rejection of cardiac allografts in $Tbet^{-/-}$ mice (that are unable to mount a Th1 response) and were able to prevent this accelerated rejection by neutralisation of IL-17[75].

Naïve $CD4^+$ T cells exposed in vitro to antigen in the presence of TGF-β alone tend to develop into regulatory T cells (Treg; discussed in detail later), but with the addition of IL-6, they can be induced to differentiate into the Th17 effector phenotype[76]. An array of additional mediators is also capable of augmenting or facilitating Th17 differentiation, many of which are released as a result of IRI, including IL-23[77], prostaglandin E2[78], HMGB1[79] and TLR ligands[80,81]. A causative role for Th17 lymphocytes in mediating rejection has now been shown in multiple transplant models including murine skin[82], heart[83,84], trachea[85] and lung[86]. Clinically, elevated serum levels of IL-17 have been observed during episodes of acute rejection following liver transplantation[87] and the presence of Th17 cells within renal allografts has also been associated with accelerated rejection[88].

Th17 lymphocytes not only compete with Treg for the TGF-β necessary for their respective differentiation, but they also produce IL-21, which directly suppresses the transcription factor Foxp3 that is necessary for Treg development[89]. This, and possibly a number of other mechanisms, likely underlie the relative resistance of Th17 cells to Treg-mediated suppression[90]. The action of TGF-β on T cells is antagonised by IFN-γ and IL-4, therefore both of these cytokines can suppress Th17 differentiation and potentially afford allografts some protection from rejection as a

result[76]. Interestingly, experiments with a murine kidney transplant model have shown prolonged allograft survival in IL-17$^{-/-}$ recipients, although in this setting, protection was associated with reduced, rather than elevated, IFN-γ production. This effect could be recapitulated in WT animals with antibody-mediated neutralisation of IL-17 and suggests that IL-17 can also facilitate Th1-mediated rejection (in contrast to the reciprocal situation, where Th1 responses likely suppress Th17 through production of IFN-γ)[91].

In summary, Th17 lymphocytes appear to have an unequivocally detrimental effect on transplanted allografts; Th1 lymphocytes are most often harmful, but the IFN-γ they produce can sometimes be beneficial in suppressing Th17 differentiation; Th2 lymphocytes are often beneficial in respect of producing IL-4 that suppresses both Th1 and Th17 differentiation, but they are also capable of mediating acute rejection indirectly and probably contribute to graft dysfunction in the long term as a result of fibrosis.

1.1.3.7 CD8$^+$ T cells

CD8$^+$ cytotoxic T cell activation requires a number of steps: following recognition of MHC class I complexes on allogeneic cells, co-stimulation is required from either a highly-activated mature dendritic cell or, more usually, with additional input from an activated CD4$^+$ T cell. In this arrangement, activated CD4$^+$ T cells express CD40 ligand (CD40L), which is detected by CD40 on the surface of APCs and thereby licences the APC to provide additional costimulation to the bound CD8$^+$ cell[35]. Upon activation, CD8$^+$ T cells can mediate killing through release of cytotoxic molecules (perforin and granzyme B) and upregulation of Fas ligand[92,93]. Prevention of CD8$^+$ T cell activation via blockade of CD40 considerably prolongs the survival of rat cardiac allografts[94] and, clinically, kidney allograft rejection has been found to correlate with urine concentrations of perforin and granzyme B mRNA[95].

1.1.4 Regulatory Cells and Mechanisms

Many of the component cell types that play a key role in rejecting an allograft (e.g. macrophages, DCs, B cells and T cells) are also capable of developing a regulatory phenotype and suppressing rejection[96]. In the absence of exogenous immunosuppressive agents, the balance between tolerance and rejection is determined by the comparative frequency and potency of effector vs. regulatory cells and the status of this equilibrium therefore fundamentally determines graft outcome in the long term.

1.1.4.1 Macrophages

Recipient macrophages infiltrate into allografts in the early post-transplantation period and are a component of the innate immune response to allograft damage. Classically, this can result in initiation and amplification of the alloimmune response as described above. However, macrophages can adopt an 'alternatively activated' phenotype which inhibits the release of inflammatory cytokines. Alternatively activated macrophages (AAM) are induced by the Th2 cytokines IL-4 and IL-13, and are closely associated with helminth infections and promotion of wound healing responses[68]. Additionally, in response to IL-10, macrophages appear to effectively skew the adaptive immune response away from a Th17 phenotype and can thereby ameliorate pathology such as colitis[97]. In the context of transplantation, AAM (and possibly type 2 immune responses in general) can be beneficial in the early post-transplantation period by inhibiting pro-inflammatory cytokines, but may contribute to chronic graft dysfunction in the longer term as a consequence of fibrosis.

A further population of 'regulatory macrophages' (distinct from AAM) is characterised by production of IL-10[98]. The significance of immune regulation exerted by macrophages has been demonstrated experimentally in a murine model of allogeneic haematopoietic cell transplantation, where antibody-mediated depletion of macrophage populations results in aggravated graft vs. host disease (GvHD) and an increase in mortality[99]. Further, one clinical pilot study infused donor-derived

regulatory macrophages into two patients prior to kidney transplantation from a living donor[100]. No adverse effects were experienced and both patients were subsequently maintained on minimal immunosuppression therapy with no signs of rejection in three years of follow up. Further investigation of the potential therapeutic role for regulatory macrophages will be conducted as one arm of a major multicentre clinical trail (the ONE study, NCT02129881).

1.1.4.2 Tolerogenic dendritic cells

The term 'dendritic cell' describes a heterogeneous group of monocytic phagocytes with multiple subtypes and functions[101]. Maturation and development of DCs is largely influenced by their surrounding cytokine environment and two main subtypes exist: conventional (or myeloid) DCs and plasmacytoid DCs (pDCs)[102]. Immature DCs exhibit relatively low expression of MHC and co-stimulatory molecules and can therefore present alloantigens in a 'tolerogenic' fashion; infusion of immature DCs in experimental murine models has been shown to be effective in inducing tolerance of allogeneic vascularised cardiac grafts[103] and full thickness skin grafts[104]. Plasmacytoid DCs (pDCs) can also be effective in promoting allograft tolerance through generation of Treg, suggesting that maturity of DCs does not necessarily mandate development of a pro-inflammatory phenotype[105].

The prospect of therapeutic infusion of DCs in a clinical setting clearly presents a risk of enhancing alloantigen presentation and augmenting the adaptive immune response (particularly within the inflammatory cytokine milieu inside a rejecting allograft). It has been suggested that co-treatment with pharmacological agents to inhibit co-stimulation might be an effective strategy to negate this risk[96].

1.1.4.3 Regulatory T cells

$CD4^+$ regulatory T cells (Treg) are widely regarded as the single most important cell type in mediating peripheral tolerance[106] and are comprised of two main subtypes.

The predominant form of regulatory T cells is identified by the transcription factor Foxp3 (described herein as 'Treg') and is further classified according to two origins: thymic Treg (tTreg) constitutively express Foxp3 and have the predominant role of maintaining tolerance to self antigens, whilst peripheral Treg (pTreg) develop in the periphery from naïve $CD4^+$ cells that are exposed to antigens under tolerogenic conditions (for example by immature DCs with low levels of co-stimulation). A second group of $CD4^+$ regulatory T cells that do not express Foxp3 has also been identified and named as Type I regulatory cells (Tr1)[107]. Tr1 cells are generated in the periphery upon antigen stimulation in the presence of IL-10 and utilise many of the same regulatory mechanisms as Treg[108], but the two may have spatially and temporally distinct roles in achieving allograft tolerance in the *in vivo* setting[109].

Treg are crucial for physiological immune homeostasis and their absence leads to severe autoimmunity, which is universally fatal in 'scurfy' mice that lack Foxp3 expression[110] and manifests as a life-limiting multisystem disorder in humans – the immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX)[111,112]. In transplantation, Hall *et al* first identified Treg in rats that had become tolerant of allogeneic cardiac grafts; they found that these cells showed alloantigen-specific unresponsiveness *in vitro* and then demonstrated that they could induce tolerance in rats with newly transplanted allografts[113]. Since then, Treg have been shown to mediate tolerance in a vast array of experimental murine allograft models including islet cells[114], heart[115], skin[116] and haematopoietic stem cell transplantation (HSCT)[117].

Investigation of some of the small number of patients who have become tolerant of liver allografts following withdrawal of immunosuppression has also revealed larger populations of Treg in peripheral blood compared to patients who went on to reject their grafts[118]. However, Treg do also have the potential to cause harm by permitting neoplastic cells to evade anti-tumour immunity[119] and preventing sterile immunity in certain viral infections[106]; the long term effects of artificially manipulating Treg populations *in vivo* are therefore unknown.

Treg appear to mediate suppression via four synergistic mechanisms: release of inhibitory cytokines, direct cytolysis, metabolic disruption and modulation of DC maturation and function[106]; each of these mechanisms has been shown to be effective and the relative importance of one over the others remains incompletely resolved.

Treg actively secrete IL-10, TGF-β and IL-35. Of these, IL-10 secretion is common to multiple regulatory cell types and is an essential (non-redundant) mediator of immunoregulation in a wide variety of inflammatory conditions[120]. The IL-10 receptor is most highly expressed on macrophages and DCs, but T cells, B cells and many others are also targets. Ligation of the IL-10 receptor leads to activation of signal transducer and activator of transcription 3 (STAT3), which blocks inflammatory signalling (especially signalling from Toll-like receptor stimulation)[120]. Anti-inflammatory actions of IL-10 include suppression of inflammatory cytokines, chemokines and nitric oxide release from macrophages[121], suppression of both Th1 and Th2 responses, and stimulation of $CD4^+$ cells to produce more IL-10 in a positive feedback manner[122]. TGF-β plays a critical role in the generation and maintenance of iTreg and is discussed further in Section 1.5. Finally, IL-35 is also constitutively expressed by Treg and is an important mediator of suppression. IL-35 can suppress T cell proliferation directly, but its overall effects more likely result from influencing T cell differentiation including suppression of Th1/Th17 phenotypes, whilst enhancing the suppressive capacity of Treg and stimulating their proliferation and production of IL-10 and TGF-β[123].

Treg are able to directly induce apoptosis in target effector cells with cell-cell contact through production of the serine protease, granzyme A (GZMA)[124]. This method of suppression appears to be essential for effective regulatory function in specific circumstances including haematopoietic stem cell transplantation (HSCT). Transcriptional analysis from Treg of human patients undergoing HSCT has shown significantly elevated expression of granzyme A in immune tolerant patients compared to those experiencing GvHD[125] and the relevance of this relationship has been recently confirmed experimentally: whilst $GZMA^{-/-}$ Treg are highly suppressive *in vitro*, they are unable to prevent GvHD (unlike WT Treg) in a murine model of HSCT[124].

Extracellular adenosine is a physiological negative regulator of numerous immune cell types (including T cells, B cells, macrophages and dendritic cells) and its concentration can increase from metabolic changes in states of hypoxic stress, or as a result of active processes employed by Treg[126]. Cell surface expression of 'ecto-enzymes' CD39 and CD73 is an effective mechanism for increasing the local extracellular concentration of adenosine: CD39 dephosphorylates ATP to AMP and CD73 further dephosphorylates AMP to adenosine. Treg express high levels of both CD39 and CD73, and adoptive transfer experiments with $CD73^{-/-}$ Treg have demonstrated that its expression is necessary for suppression of effector cells in murine models including HSCT[127] and ischaemia-reperfusion injury[128]. At the same time, adenosine can act in an autocrine positive feedback fashion to augment the suppressive ability of Treg. Exposing Treg to an adenosine receptor agonist in culture promotes their expression of cell surface inhibitory molecules including CTLA-4[129] and PD-1[130] and enhances their ability to ameliorate ischaemia-reperfusion injury.

As mentioned above, DCs play a key role in determining the balance between inflammation and tolerance. Whilst tolerogenic DCs are able to promote Treg induction and expansion, Treg are also able to indirectly mediate tolerance by manipulating the phenotype of local DCs. Treg expression of CTLA-4 contributes to this by decreasing DC expression of CD80/CD86 costimulatory molecules, thereby reducing their ability to drive T cell activation[131]. Lymphocyte activation gene 3 (LAG3/CD223) is a type I membrane glycoprotein found on the surface of Tr1 cells; binding of LAG3 to MHC II molecules on the surface of DCs directly inhibits DC maturation and expression of co-stimulatory molecules[132]. Expression of LAG3 therefore provides a contact-dependent mechanism of inducing tolerogenic DCs, in addition to secretion of IL-10.

In most instances of allogeneic transplantation, a relatively small number of functioning nTreg and iTreg are present, but their regulatory influence is overwhelmed by the comparatively very high number of circulating effector T cells with specificity for allogeneic MHC molecules. In light of the multiple effective regulatory mechanisms at their disposal, expansion of Treg populations is an

attractive therapeutic prospect; current strategies under investigation are discussed further in Section 1.1.6.

1.1.4.4 Myeloid-derived suppressor cells

Myeloid-derived suppressor cells (MDSCs) are a notoriously heterogeneous group consisting of immature myeloid cells and myeloid progenitor cells that are known to expand during inflammation and can potently suppress T cell proliferation[133]. In the context of transplantation, MDSCs have been shown to ameliorate allograft rejection through several mechanisms[96,134]. First, MDSCs can directly suppress proliferation of B cells, T cells and NK cells. Secondly, they promote or maintain regulatory phenotypes of other immune cells including Treg, regulatory macrophages and immature DCs. And thirdly, they also secrete soluble mediators to suppress inflammation, including IL-10 and haem oxygenase-1[135], an enzyme that has been shown to ameliorate cell damage from ischaemia-reperfusion injury[136]. However, due to the diversity of settings and stimuli that elicit MDSCs, and the marked heterogeneity of their origin and phenotypic markers, a clear role in allograft tolerance has yet to emerge.

1.1.4.5 Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are multipotent stem cells that contribute to haematopoiesis in bone marrow, but also migrate into transplanted allografts following the release of inflammatory cytokines arising from the innate immune response. MSCs are capable of suppressing T cell proliferation through mechanisms similar to that of myeloid-derived suppressor cells (expansion of Treg, maintenance of DC immaturity and release of anti-inflammatory soluble mediators)[137].

1.1.5 Current Immunosuppression Regimens and Sequelae

The degree of immunosuppression required to prevent rejection differs according to the particular organ being transplanted. Skin, small bowel and lung allografts are particularly prone to rejection, whilst pancreas, heart, kidney and liver are progressively less vulnerable[138]. Specific immunosuppression regimens therefore differ accordingly, but share the same set of commonly used pharmacological agents as summarised below.

1.1.5.1 Corticosteroids

Exogenous corticosteroids have been used clinically to combat inflammation from autoimmunity since 1948[139] and remain the first line treatment for episodes of acute rejection in most centres today[140]. Corticosteroids act via a number of mechanisms to suppress inflammation, including inhibition of gene transcription leading to a reduction in prostaglandin synthesis and extra-genomic actions such as activation of endothelial nitric oxide synthase[141]. Whilst the immunosuppression achieved is effective in preventing allograft rejection, multiple serious adverse effects considerably limit clinical use. These include: diabetes, hypertension, dyslipidaemia, infection, poor wound healing, osteoporosis and accelerated cardiovascular disease. As a result, steroid therapy is usually reserved for use upon induction of immunosuppressive therapy (and rapidly tapered off), or for treatment of episodes of acute rejection, so that the cumulative dose is minimised wherever possible[142].

1.1.5.2 Purine Inhibitors

Mycophenolate mofetil (MMF) and azathioprine act via different mechanisms to inhibit purine (and therefore DNA) synthesis necessary for lymphocyte activation. Azathioprine formed the mainstay of immunosuppressive therapy (in combination with corticosteroids) in the early days of transplantation, but has now been largely superseded by MMF, which is more potent and specific. MMF blocks purine

synthesis by inhibition of inosine monophosphate dehydrogenase and specificity for B and T cells is achieved because they almost uniquely lack the ability to alternatively generate purine from nucleotide breakdown products (the purine salvage pathway)[143]. MMF does not achieve sufficient immunosuppression as a sole agent to prevent rejection; it is teratogenic and associated with adverse effects including leucopoenia, abdominal pain, diarrhoea, diabetes and dyslipidaemia.

1.1.5.3 Calcineurin Inhibitors

Calcineurin is a protein phosphatase involved in a number of cellular processes, predominantly enabling nuclear factor of activated T cells (NFAT) to translocate to the nucleus and upregulate expression of IL-2, leading to T cell proliferation[144]. Drugs that inhibit calcineurin include cyclosporine and, more recently, tacrolimus. Cyclosporine alone is capable of preventing acute rejection and has formed the basis of most immunosuppression regimens (in combination with corticosteroids) over the last 30 years. However, renal impairment secondary to interstitial fibrosis is a major complication of calcineurin inhibitors - severe renal failure affects up to 20% of non-kidney transplant recipients at 5 years[145]. Cyclosporine is also associated with additional specific adverse effects including hypertension, hirsuitism and gum hypertrophy. Tacrolimus is a macrolide with greater bioavailability and immunosuppressive potency than cyclosporine. It is also associated with fewer adverse effects overall, but does frequently cause neurological symptoms (tremor, headache, insomnia), dyslipidaemia and diabetes[146].

1.1.5.4 mTOR inhibitors

Mammalian target of rapamycin (mTOR) is a serine/threonine kinase that mediates T cell proliferation in response to IL-2 and several other cytokines. Sirolimus (rapamycin) inhibits mTOR-mediated signal transduction and the CD28-induced co-stimulatory pathway. Sirolimus is less nephrotoxic than calcineurin inhibitors, promotes induction of Treg and, in contrast to most immunosuppressive agents, has

some anti-cancer properties. Adverse effects include bone marrow suppression, dyslipidaemia, thrombocytopenia and severely impaired wound healing, which presents a particular problem in the context of recent major surgery[142].

1.1.5.5 Depleting antibodies

Antithymocyte globulin (ATG) is a polyclonal antibody raised against whole human T cells that acts on a number of fronts – preferential depletion of $CD8^+$ and $CD4^+Foxp3^-$ effector T cells compared to $CD4^+Foxp3^+$ Treg[147], induction of B cell apoptosis and modulation of DC function[148]. ATG is a highly-effective immunosuppressant but its use is complicated by multiple severe adverse effects: in the short term these include 'cytokine storm' (manifesting as fever, hypotension and pulmonary oedema); in the longer term, lymphopoenia can persist for more than 12 months and therefore lymphoma and opportunistic infections are more common than with other immunosuppressant agents[140]. As a result, use of ATG is generally restricted to patients at high risk of rejection and for instances of acute rejection resistant to steroid therapy.

Alemtuzumab is a humanised monoclonal antibody to CD52, which is abundantly expressed on the surface of T cells and B cells. Alemtuzumab is used for similar indications to ATG and is associated with fewer adverse events, but does carry a particular risk of *de novo* autoimmune conditions[141].

1.1.5.6 Non-depleting antibodies and novel agents

Basiliximab is a monoclonal antibody to CD25, the α chain of the IL-2 receptor, which is expressed on activated T cells. This degree of specificity seems to result in basiliximab exhibiting fewer adverse effects compared to alemtuzumab (anti-CD52), but with the risk of more frequent episodes of acute rejection[149]. As CD25 is also expressed on Treg, basiliximab may also inhibit the development of tolerance.

Belatacept is a fusion protein comprised of the extracellular component of CTLA-4 combined with the Fc domain of IgG1. Acting as an exogenous source of CTLA-4, it regulates T cells by effectively inhibiting co-stimulation through CD80/86. Belatacept can be used for maintenance immunosuppression, with the advantage of not causing any nephrotoxicity. However, its use is associated with bone marrow suppression, dyslipidaemia and a particularly high risk of *de novo* cancers[149].

1.1.5.7 Generic adverse effects

Patients suffering from end-stage kidney disease (ESKD) show a slightly higher incidence of a variety of cancers in addition to those that are known to cause renal impairment (kidney, urinary tract, thyroid and myeloma)[150]. The increased risk of malignancy is seen both before and after commencing dialysis and may be partially attributable to uraemic immunosuppression[151]. Following transplantation, currently employed immunosuppression regimens contribute to a 100-fold increase in the relative risk of skin cancer and up to 5-fold increase in the relative risk of other cancers[152,153]. This pattern of malignancy is similar to, but greater than, the variety seen with immunocompromise arising through infection with Human Immunodeficiency Virus (HIV)[154] and includes multiple neoplasms with a known viral aetiology such as non-Hodgkin lymphoma, Kaposi's sarcoma and non-melanomatous skin cancers[150]. Cancer risk with immunosuppression appears to be cumulative and therefore presents a particular challenge to the paediatric transplant recipient population[155].

As outlined above, significant cardiovascular risk factors (hypertension, diabetes, dyslipidaemia) are frequent complications of multiple immunosuppressive agents. As a result, cardiovascular mortality now accounts for the fact that the life expectancy of transplant recipients still falls short of the general population[141]. The repertoire of immunosuppressive agents described here has remained largely unchanged in recent years; novel therapeutic strategies are urgently required.

1.1.6 Emerging Therapies Under Investigation in Transplantation

Allograft tolerance, defined as durable antigen-specific unresponsiveness in an immunocompetent host[156], presents the 'Holy Grail' scenario of achieving the benefits of organ transplantation without the need for immunosuppression and all of the harmful sequelae it encompasses.

Despite successful achievement of allograft tolerance in murine experimental models more than 60 years ago[157], translation to routine clinical practice has remained elusive. Recent developments in the understanding of regulatory cell populations have however allowed some ground for optimism[96]. In particular, a recent trial of combining kidney transplantation with a simultaneous bone marrow transplant from single HLA-mismatched donors has allowed for successful withdrawal of all immunosuppression in four of five patients[158]. With this technique, patients developed transient mixed chimerism and lasting specific alloantigen unresponsiveness as a result. Using the same technique, Scandling *et al.* have independently demonstrated similarly impressive outcomes with HLA-matched kidney transplantation: from a cohort of 16 patients, 8 have achieved rejection-free avoidance of immunosuppressive medication for more than 1 year, and a further 4 patients are in the process of withdrawal from medication[159]. Although many would consider simultaneous bone marrow transplantation to present an unacceptable level of complexity and risk in the pursuit of solid-organ allograft tolerance[160], these important studies have shown that it is possible to achieve durable intragraft regulatory cell populations and successful allograft tolerance in a clinical setting as a result.

Less invasive approaches to the achievement of clinical tolerance have focussed on the potential role for cellular therapy. The ONE Study (currently in progress) is a multicentre clinical trial investigating multiple cellular therapies in parallel: Treg, tolerogenic dendritic cells and regulatory macrophages[161]. Of these, Treg therapeutic strategies have received by far the most attention.

The ability of adoptively transferred Treg to mediate indefinite tolerance of murine allografts has now been demonstrated in multiple transplant models including pancreatic islet[162], skin[163] and heart[164]. Expectations of successful translation of Treg therapy into the clinical setting have been high and preliminary clinical trials have now been completed in graft-versus-host disease[165] and hematopoietic stem cell transplantation[166] with modest but encouraging results.

However, a number of obstacles and concerns persist. First, Good Manufacturing Practice (GMP)-compliant *ex vivo* expansion of Tregs for subsequent reinfusion is a highly specialized process at a cost of approximately £30,000 ($45,000) per patient[167]. Even if this level of funding could be justified, the infrastructure and highly qualified personnel required are likely to limit translation into routine clinical practice.

Secondly, due to the lack of a unique human regulatory T cell surface marker, accurate identification of Treg populations remains imperfect. The optimal approach is with fluorescence-activated cell sorting (FACS), with selection of, for example, $CD4^+CD25^+CD127^{lo}$ cells[166]. However, GMP-compliant FACS isolation of Treg for clinical therapeutic use is available at very few centres throughout the world and therefore current preliminary clinical trials are utilising magnetic cell sorting techniques, consequently resulting in Treg populations of a lower purity[168,169]. Currently employed regimens include depletion of CD8+ cells, followed by enrichment of $CD25^+$ cells and subsequent expansion via stimulation with anti-CD3 and anti-CD28 monoconal antibody-coated beads and the addition of IL-2 with rapamycin to selectively inhibit proliferation of non-Treg cells[169]. Whilst this technique limits alloreactive effector T cells to a very small percentage, it is likely that the few remaining are highly activated, and the long-term impact of their infusion into a transplant recipient is unpredictable[169].

Thirdly, concern remains over the question of whether isolated Treg maintain their regulatory phenotype following re-infusion, particularly in the context of an inflammatory environment. Alloantigen-specific iTreg offer the potential advantages of high functional suppressive ability and a specificity of action that might lower the

risk of side effects such as early viral reactivation (observed in trial of Treg therapy in haematopoietic stem cell transplantation[170]) and the potential risk of neoplasia with non-specific Treg therapy. However, whilst *ex vivo* expansion of alloantigen-specific iTreg driven by allogeneic dendritic cells may have advantages of specificity and potency, loss of Foxp3 expression (and therefore regulatory phenotype) has been reported[171]. This poses a risk of infusing a population of cells that effectively revert to allograft-specific effector T cells, and the ability or otherwise to treat this scenario with conventional immunosuppression is unknown[172]. Therapeutic infusion of nTreg and iTreg comprise two separate arms of the ONE Study that is currently underway (NCT02129881).

Finally, in the long-term it is unknown whether Treg-mediated immunosuppression might present risks of infection or neoplasia comparable to those of current non-specific immunosuppression regimens. To date, four clinical trials of Treg therapy have been published: three investigating prevention or treatment of graft vs host disease (GvHD)[165,173,174] and one for treatment of type I diabetes[175]. Early follow-up has provided some degree of reassurance, with no adverse events reported other than a slightly increased incidence of viral reactivation in the context of GvHD[170]. However the longest follow-up period that has been reported is only 12 months[175] and, particularly regarding a potential long-term risk of malignancy, it may be very difficult to determine a follow-up period wherein this question can be answered definitively. In short, Treg cellular therapy is an attractive potential therapeutic strategy that has advanced rapidly in recent years, but many questions and logistical barriers still exist, such that translation to routine clinical practice is by no means guaranteed.

1.2 Autoimmunity and the Hygiene Hypothesis

1.2.1 Autoimmunity

Autoimmune disease encompasses a diverse group of conditions characterised by abnormal immune reactivity in association with auto-reactive B and T cells that results in damage to host tissue. Disease ranges from the organ-specific (e.g. autoimmune thyroiditis and type I diabetes) to more general multi-system disorders (e.g. systemic lupus erythematosus). In a minority of patients, a specific aetiology or precipitant is determinable, such as a genetic lack of regulatory T cell activity in IPEX syndrome leading to multi-system autoimmunity[111], or preceding *Streptococcal* infection leading to rheumatic fever[176]. In most cases, aetiology is thought to be multifactorial, with contributing risk factors of genetic susceptibilities and environmental exposures accumulating over time to manifest as clinical disease in adulthood[177,178].

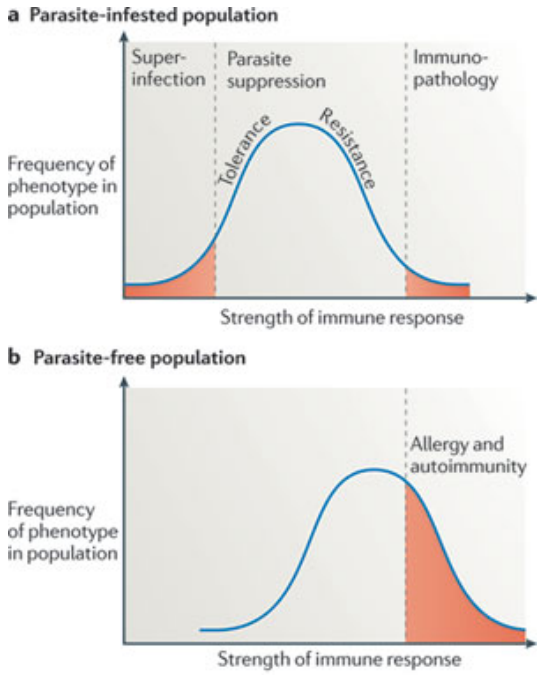
Multiple sclerosis is an inflammatory disorder of the central nervous system that results in focal areas of axonal demyelination and currently represents the leading cause of neurological disability in young adults worldwide[179,180]. Whilst the precise aetiology of the disease is unknown, multiple factors combine to justify its classification as an autoimmune condition, including characteristically activated T cells in focal lesions of the central nervous system[181], the presence of oligoclonal IgG in cerebrospinal fluid[182] and differing susceptibility to the disease between distinct HLA alleles[183].

The prevalence of autoimmune diseases in general has been increasing in developed countries[179,184], to such an extent that 3% of the general population of the United States is now affected[185]. Indeed, the rapidity of rising incidence is outwith a timescale that could be explained by genetic evolution and therefore strongly points towards a causative environmental change. A number of aetiological hypotheses have been proposed including changes in UV light exposure[186], vitamin D levels[187], geographical latitude[188], month of birth[189], novel inciting infections[190], environmental

toxins[191] and a relatively sudden change in sanitation and exposure to infection (the 'hygiene hypothesis') [192].

1.2.2 The Hygiene Hypothesis

The hygiene hypothesis has developed into a number of forms over time. It began in 1968 with the observation of a low incidence of rheumatoid arthritis in an area of West Africa where helminth infections were endemic[193]. Subsequently (and more conspicuously), Strachan proposed a causal link between household size in childhood and the incidence of hay fever in later life, suggesting that children from large families were exposed to a greater range of pathogens, which 'educated' the immune system and prevented inappropriate overactivity in later life[192,194]. Continuing this theme is the 'Old Friends Hypothesis[195]', that certain Palaeolithic commensal bacteria and helminth parasites have co-evolved with humans for thousands of years until their sudden widespread eradication in developed countries with improvements in sanitation and the advent of effective anti-helminthic therapy in the 1930s, a time that closely corresponds with the onset of an epidemic of allergic and autoimmune conditions in the same geographical regions. Rook and others proposed that the sudden removal of helminth-derived immunoregulation tipped the balance towards overactive immunity manifesting as atopy and autoimmunity[196].

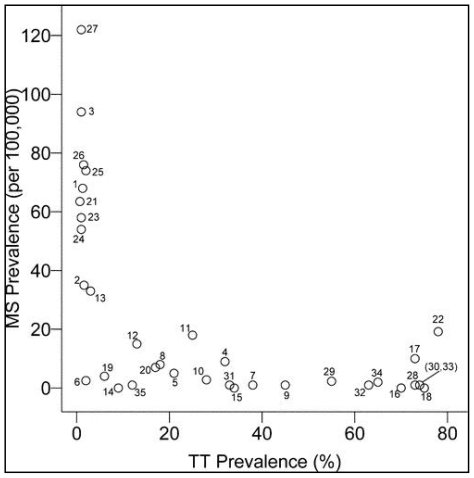


Within the diverse spectrum of genetic polymorphisms of the human population, many non-coding alleles exert some influence on the general level of immune responsiveness. Evolution in the context of prevalent helminth infections (a) will compensate for the suppressive effect exerted by the parasites through positive selection of the most appropriate alleles; extremes of under- or over-responders are rare. Since the relatively sudden eradication of the helminth-derived suppressive effect in the modern-day environment (b) more pro-inflammatory genotypes that would have previously provided an appropriate level of immune responsiveness may now be prone to pathological over-activity in the form of allergic and autoimmune conditions.

Figure 1-4 Evolution of Immune Responsiveness with Parasite Immunomodulation. Adapted from Allen & Maizels (2011)[197].

The 'Old Friends' hypothesis presents a clearly logical rationale: in the face of an evolutionary imperative, helminths have developed powerful mechanisms to modulate and suppress the immune responses of their host; over time, the host has adapted to this immunosuppression with a more active level of baseline immunity and sudden removal of the helminths' suppressive influence tips the equilibrium towards harmful overactive immunity[197]. A considerable body of evidence now exists in support of this premise. Helminths chronically infect more than one quarter of the world's population[198] and their geographical prevalence bears a strikingly inverse correlation to that of multiple sclerosis (MS)[184,196]. Further to this, Fleming & Cook determined a quantitatively inverse relationship between prevalence of MS and the helminth *Trichuris trichiura*, with the observation that MS prevalence falls steeply when a threshold of approximately 10% prevalence of *Trichuris trichiura* infection is exceeded (Figure 1-4)[199]. The same dichotomous relationship has been found with other helminth species (*Necator americanus* and *Ancylostoma*

duodenale)[200] and to exist to a similar extent between populations within individual countries[199].



The prevalence of MS (patients per 100,000 general population) and T trichiura (percentage of surveyed population infected) by country or region.

North America: northern United States (1), southern United States (2), Canada (3). Latin America: Brazil (4), Mexico (5), Paraguay (6), Honduras (7), Cuba (8), Jamaica (9), Panama (10), Argentina (11), Chile (12). Oceania: Australia (13). Africa: Ethiopia (14), Kenya (15), Cameroon (16), Nigeria/Ghana (17), Ivory Coast/Senegal (18). Middle East: Iran/Iraq (19), Lebanon/Jordan (20), Jerusalem-Jewish (21), Jerusalem-Arab (22). Europe: Italy (23), Poland (24), Belgium (25), East Germany (26), United Kingdom (27). Asia: Indonesia (28), Japan (29), Korea (30), Malaysia (31), Taiwan (32), Philippines (33), China (34), Central India (35).

Figure 1-5 - Dichotomous relationship between *Trichuris trichiura* and multiple sclerosis. Adapted from Fleming & Cook (2006)[199]

In pursuing a causal relationship, van den Biggelaar *et al* performed skin allergen testing in 520 Gabonese schoolchildren and found a significantly lower prevalence of positive skin reactions amongst children with coincidental urinary schistosomiasis[201]. Peripheral blood T cells from infected children were found to produce significantly higher levels of IL-10 in response to parasite antigen stimulation, and serum IL-10 was negatively associated with allergen reactivity. A follow-up study prospectively randomised 317 schoolchildren from the same region to receive pharmacological treatment to clear helminth infection (praziquantel and mebendazole)[202]. Repeated skin allergen testing at 3-monthly intervals revealed a significant increase in sensitivity to house dust mite following clearance of helminth infection in the treatment group, thus indicating a causal relationship for the first time.

There is also some evidence to suggest that maternal helminth infection may afford some protection from future atopy to the unborn foetus *in utero*. Webb *et al*

conducted a double-blind, placebo-controlled randomised controlled trial of 2,507 pregnant women in Uganda to investigate the hypothesis that antihelminthic treatment in pregnancy might improve the efficacy of subsequent childhood immunisations[203,204]. After five years of follow-up, the study failed to show any effect on recall responses to Bacille Calmette Guérin (BCG) or tetanus immunisations. However, children born from mothers randomised to receive antihelminthic treatment (albendazole) experienced a significantly higher incidence of (physician-diagnosed) eczema[205]. This suggests that, in some cases at least, helminth infection may be not only safe in pregnancy, but beneficial in reducing atopic conditions in offspring as well.

Evolutionary selection may influence the host as well as the parasite. Investigation of a population of wild Soay sheep on the island of St Kilda off the coast of Scotland revealed an inverse correlation of antibody responses to *Teladorsagia circumcincta* (a prevalent parasitic nematode) and reproductive fitness[206]. This relationship may underlie a selection bias of hosts that tolerate helminth infection.

1.2.3 Observational Studies of Autoimmunity and Helminth Infection

In Argentina, Correale and Farez initiated a small prospective double cohort study assessing the clinical disease course and radiological features of 24 patients with established MS – 12 patients with environmentally-acquired asymptomatic helminth infection and 12 matched, non-infected patients as a control cohort[207]. Over a period of 4.5 years, study subjects underwent a comprehensive neurological examination every three months, magnetic resonance imaging (MRI) brain scan every six months and further specific immunological investigations towards the end of the study period. The results are convincing: in 55.8 months, 3 episodes of clinical relapse were seen in the helminth-infected cohort, compared to 56 relapses in the uninfected control group (median annualised relapse rate of 0 vs. 1.10, $p < 0.0001$). Radiological findings support the clinical results – in the infected cohort, 6 out of 12

patients showed a change, with a total of 14 new or enlarging MRI lesions, compared with radiological changes in all patients of the non-infected cohort, with 164 new or enlarging MRI lesions in total. Mechanistically, peripheral blood analysis revealed a significantly greater percentage of $CD4^+Foxp3^+$ Treg in the helminth-infected cohort, in addition to elevated levels of the regulatory cytokines TGF-β and IL-10, and decreased expression of pro-inflammatory cytokines IFN-γ and IL-12.

In 2011, Correale and Farez published an update on the original study above after 7.5 years of follow-up[208]. After 63 months of monitoring, 4 out of the cohort of 12 patients with helminth infection suddenly developed symptoms related to the infection, including abdominal pain and general malaise, and were therefore treated with anti-helminthic therapy. This intervention provided some additionally illuminating insights – whilst the 8 untreated patients with helminth infection continued to experience a very low clinical relapse rate (0.06 episodes per patient/year), those who had pharmacologically cleared the parasite infection saw a dramatic rise in their relapse rate of MS symptoms (1.1 episodes per patient/year, $p = 0.007$), a level comparable to patients who had never experienced any helminth infection. Despite this study's small sample size and lack of randomisation or blinding, the dramatic change in reported clinical disease course was very closely replicated by radiological evidence of additional new or enlarging lesions (figure 1-5), a decrease in $CD4^+Foxp3^+$ Treg and a corresponding 'switch' in myelin basic protein (MBP)-specific cytokine profile (decreased IL-10 and TGF-β; increased IL-12 and IFN-γ).

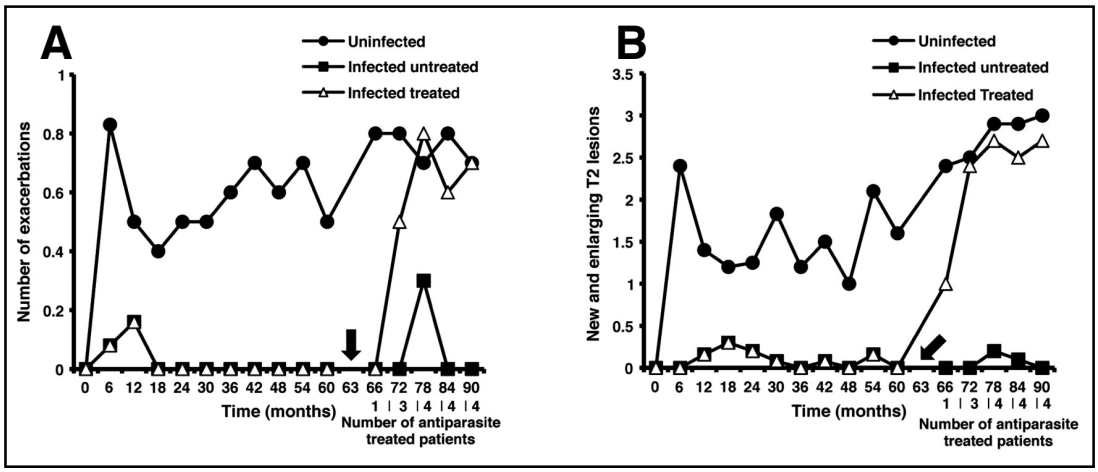


Figure 1-6 - Number of exacerbations (A) and changes in MRI parameters observed over time (B) in parasite infected MS patients, uninfected MS patients, and parasite infected MS patients under anti-parasite treatment. Arrows indicate onset of treatment with anti-parasite drugs. For all panels, data shown represents average values observed at each time point. Adapted (with permission) from Correale and Farez (2011)[208]

1.2.4 Animal Studies of Autoimmunity and Helminth Infection

The impact of helminths on autoimmunity has been investigated in animal models of several diseases including type I diabetes[209], Graves' hyperthyroidism[210] and rheumatoid arthritis[211], but multiple sclerosis has received the most attention and will form the basis of further review here. Several experimental animal models have been designed to replicate multiple sclerosis, including experimental autoimmune encephalomyelitis (EAE)[212], viral-induced demyelination[213] and the neurotoxic cuprizone model[214]. Whilst none of these are perfect surrogates of clinical MS, EAE is thought to capture many of its immunological features, and is the most frequently cited, featuring in almost 20% of all MS-related research publications[215].

The collective term EAE encompasses a number of models which broadly involve either immunisation with myelin antigens (active EAE), or adoptive transfer of myelin-specific effector T cells (passive EAE), such that demyelination (and a characteristic clinical phenotype) develops as a result of inflammation in the CNS. The pathogenesis of active EAE has two distinct phases: an induction phase

(immunisation, presentation of myelin antigen by DCs, activation and expansion of myelin-specific T cells) and an effector phase (migration of myelin-specific T cells into the CNS, recognition of cognate antigen and T cell activation with secondary activation of microglia and macrophages, along with the onset of clinical disease).

One particular difficulty that arises in attempting to extrapolate findings from EAE to MS is that the experimental model often produces a single episode of disease, in contrast to clinical MS, which is typically characterised by multiple episodes of relapse followed by remission with incomplete recovery. The natural history of disease is also clearly very different – modelling an uncertain spectrum of events over a prolonged period that eventually lead to MS with a very precise time point of immunisation in EAE. The possibility of prophylactic treatment therefore exists in EAE that does not for MS.

To date, a total of 14 published studies have investigated the effect of treatment with helminths, helminth products or helminth-induced cell populations on the pathophysiology of EAE (outlined in table 1-1 below). All except one of these studies has shown a beneficial effect with helminth treatment. The exception (Chiuso-Minicucci *et al.*[216]), showed that *Strongyloides venezuelensis* had no impact on the disease course of EAE in Lewis rats. This may be an example of helminth-specific variation in immunomodulatory capability. However, since Lewis rats are non-permissive of sustained *S. venezuelensis* infection[216,217] the finding of an unchanged EAE course may represent inability of the parasite to establish rather than failure of the parasite to mediate an effect *per se*.

Reference	EAE model	Species Strain	Helminth Treatment	Effect on Clinical Disease Course
Treatment with helminth infection				
Donskow-Łysoniewska *et al.* (2012)[218]	EAE - pMOG35–55	Mouse - C57BL/6	*Heligmosomoides polygyrus* (21 days after pMOG immunization)	Reduction in established disease 2 days after infection
Reyes *et al.* (2011)[219]	EAE - pMOG35–55	Mouse - C57BL/6	*Taenia crassiceps*	Delayed onset and reduced maximal severity
Chiuso-Minicucci *et al.* (2011)[216]	EAE - MBP	Rat - Lewis	*Strongyloides venezuelensis*	No effect*
Wu *et al.* (2010)[220]	EAE - pMOG35–55	Mouse - C57BL/6J	*Trichinella spiralis*	Delayed onset and reduced maximal severity
Gruden-Movsesijan *et al.* (2010)[221]	EAE – SC homogenate	Rat- DA	*Trichinella spiralis*	Reduced maximal severity
Walsh *et al*. (2009)[222]	EAE - pMOG35–55	Mouse - C57BL/6	*Fasciola hepatica*	Reduced maximal severity
La Flamme *et al*. (2003)[223]	EAE - pMOG35–55	Mouse - C57BL/6J	*Schistosoma mansoni*	Reduced incidence and delayed onset

Table 1-1 Studies of EAE and helminth therapy (adapted and updated from Tanasescu (2014)[224] and Hasseldam (2013)[225]). pMOG – Myelin Oligodendrocyte Glycoprotein peptide; SC – spinal cord; DA – Dark Agouti; PLP – proteolipid protein.

Reference	EAE model	Species Strain	Helminth Treatment	Effect on Clinical Disease Course
Treatment with helminth-derived products				
Radovic *et al.* (2015)[226]	EAE – SC homogenate	Rat - DA	*Trichinella spiralis* ES products	Delayed onset and reduced maximal severity
Kuijk *et al.* (2012)[227]	EAE - pMOG35–55	Mouse - C57BL/6	Soluble products from: *Trichuris suis, trichinella spiralis* and *Schistosoma mansoni*	Reduced maximal severity
Zhu *et al.* (2012)[228]	EAE - pMOG35–55	Mouse - C57BL/6	LNFPIII (synthetic glycan from *Schistosoma mansoni*)	Reduced maximal severity
Zheng *et al.* (2008)[229]	EAE - pMOG35–55	Mouse - C57BL/6	*Schistosoma japonicum* soluble egg antigen	Reduced maximal severity
Sewell *et al.* (2003)[230]	EAE – PLP135-151	Mouse - C57BL/6 and SJL	*Schistosoma mansoni* ova (intraperitoneal)	Reduced maximal severity
Treatment with helminth-induced cells				
Sofronic-Milosavljevic *et al.* (2013)[231]	EAE – SC homogenate	Rat - DA	Adoptive transfer: *Trichinella spiralis* ES-treated DCs	Reduced maximal severity
Wilson *et al.* (2010)[232]	EAE - pMOG35–55	Mouse - C57BL/6	Adoptive transfer: *Heligmosomoides polygyrus*-induced CD19+CD23hi B cells	Reduced maximal severity

Table 1-1 (continued) Studies of EAE and helminth therapy (adapted and updated from Tanasescu (2014)[224] and Hasseldam (2013)[225]). pMOG – Myelin Oligodendrocyte Glycoprotein peptide; SC – spinal cord; DA – Dark Agouti; PLP – proteolipid protein.

Although the EAE models, helminths and individual treatment conditions of the studies outlined above varied markedly, a number of summative conclusions can be drawn. First, effective helminth therapy generally involved treatment prior to induction of EAE, with the notable exception of Donskow-Łysoniewska *et al*, who showed a dramatic improvement in established disease 48 hours after infection with *H. polygyrus*[218]. This may be indicative of a crucial role for helminth-derived immunomodulation early in the disease process, such as antigen presentation and/or co-stimulation. Indeed, Sofronic-Milosavljevic *et al* showed that excretory-secretory (ES) products from *Trichinella spiralis* were able to suppress maturation of DCs and that adoptive transfer of these cells was able to ameliorate the severity of EAE in rats[231]. However, the necessity for pre-treatment in the EAE model is not necessarily indicative of a bar to clinical translation. A treatment that can prevent EAE from developing may be beneficial in a clinical setting by preventing future relapses of MS and, as a result, cumulative disability in the long term.

Another recurring feature of helminth infection with EAE is of a change in the antigen-specific cytokine profile of splenocytes or inguinal lymph node cell populations upon restimulation, in the form of a reduction in IFN-γ and IL-17, and an increase in IL-4, IL-5 and IL-10. Interestingly, this switch to a 'modified Th2' cytokine response was also observed in mice that had received *Trichinella spiralis* ES-treated DCs[231], suggesting that the change in cytokine profile is as a result of active mediators secreted by the helminth, rather than solely as a response to tissue damage arising from live infection. To evaluate the mechanistic significance of this finding, Sewell *et al* demonstrated that treatment with *Schistosoma mansoni* ova resulted in a reduced severity of disease in WT mice, but that this protection was completely lost in STAT6$^{-/-}$ animals (which are unable to mount a Th2 response)[230].

A number of further illuminating mechanistic insights were provided in a study of the role of *Fasciola hepatica* infection on EAE by Walsh *et al*[222]. *F. hepatica* is associated with a multicellular infiltration of the peritoneal cavity including DCs, macrophages, eosinophils and CD4^{+} T cells. Analysis of the DC population by flow cytometry revealed an immature (tolerogenic) phenotype with significantly lower expression of CD80, CD86, CD40 and MHC class II in comparison to uninfected

control mice. Additionally, both DCs and macrophages isolated from the peritoneal cavity of infected mice were found to express high levels of IL-10 and TGF-β. Subsequently, infection of WT and IL-$10^{-/-}$ mice with *Fasciola hepatica* revealed identical reductions in both EAE severity and MOG-specific IFN-γ and IL-17 production by splenocytes, suggesting that IL-10 is not the mediator for suppressing pathology. However, when *F. hepatica*-infected WT animals were treated with a TGF-β neutralising antibody (vs. control IgG), protection from EAE afforded by the parasite was completely reversed, thus confirming a critical role for TGF-β in *F. hepatica*-mediated protection from EAE.

Many helminth species are capable of inducing $Foxp3^{+}$ Treg[233-237] and Treg have separately been shown to potently suppress EAE[238]; notably, the one study detailed above that did not find any evidence of protection from EAE with helminth infection, also found no expansion of Treg in the infected group, which may account for the lack of protection[216]. Other regulatory cells have been shown to play a fundamental role as well: Wilson *et al* demonstrated that $CD19^{+}$ B cells in the mesenteric lymph nodes of mice chronically-infected with *H. polygyrus* are able to effectively suppress EAE upon adoptive transfer into naïve recipients[232]. $CD19^{+}$ B cells transferred from *H. polygyrus*-infected IL-$10^{-/-}$ were equally suppressive, providing further evidence that IL-10 is not the key mediator of suppression in this model.

In summary, experimental studies with EAE strongly support the accumulating epidemiological and observational human studies suggestive of a role for helminths in ameliorating autoimmune disease. The precise pathophysiology of helminth-mediated protection has yet to be fully elucidated, but some mechanistic insights have emerged: TGF-β, Th2 polarisation, tolerogenic DCs and B cells with a regulatory phenotype all appear to be non-redundant mechanisms of protection whilst IL-10 does not play an essential role in helminth-dependent dampening of autoimmunity.

1.2.5 Challenges Facing Therapeutic Helminth Infection

The observational data summarised above presents a compelling case for progression to interventional clinical studies including randomised controlled trials and, accordingly, 17 clinical trials are now underway or completed[239]. However, the proposal of experimentally infecting patients with live helminths engenders a wide range of regulatory, logistical and scientific challenges, such that its unequivocal validation as a beneficial and viable therapy remains elusive[240].

Challenges include a heterogeneous response amongst the patient population to helminth infection that reflects the spectral nature of disease in humans. Individuals that mount a particularly weak response can develop very high worm burdens and suffer pathology as a result (e.g. local trauma, pain, anaemia)[241], whilst those that mount an unusually overactive immune response may develop a low worm burden, but potentially also suffer pathological fibrosis as a form of 'collateral damage' from an overly exuberant Th2 response[68].

Whilst the vast majority of helminth infections may persist in an asymptomatic host without any overt pathology[197], in a therapeutic context, inherent variability of worm burden presents a requirement for frequent monitoring of adverse effects and an inability to titrate dosage according to clinical disease severity (a standardised number of larvae could result in a wide range of adult worms in different patients).

To the extent that varying response to helminth infection reflect long-standing genetic polymorphisms in the host immune system, it is plausible that the trade-off between the beneficial and detrimental effects of helminths may depend upon host genotype. However, at the present time no markers have been defined that would allow the patient cohorts to be stratified for clinical trials.

Choice of helminth species is also difficult. In exploring the vast range of helminths that exist naturally, it is clear that many have a diverse range of complex immunomodulatory capabilities, and therefore likely not only that certain species will be better suited to ameliorating specific autoimmune conditions that others[242], but also that the risk of unintentionally exacerbating disease may be inconsistent and

unpredictable[243,244]. The ideal 'therapeutic helminth' conforms to several criteria[224,245]: it should be easily administered and maintain chronic infection (to avoid repeated dosing); non-pathogenic and noncontagious; localise to the host intestine without migrating to other organs and not proliferate more aggressively in hosts receiving concomitant immunosuppressive therapy. No one helminth species fulfills every criterion, two helminths most closely matching have now been used therapeutically in clinical trials: *Trichuris suis* and *Necator americanus*.

T. suis is a porcine whipworm that can colonise humans as a transient zoonotic infection[246]. It offers the advantages of a predictable life cycle (one egg releases one larva that matures into a single worm), infection confined to the host intestine and an inability to proliferate within the host[247]. However, as the infection is zoonotic, knowledge of the natural history of infection in humans is limited and therefore the possibility of systemic dissemination of parasites in a minority of cases cannot be completely excluded[224]. A further unknown is that the extent to which this species may be fine-tuned to modulating the porcine, rather than the human, immune system. Finally, infection persists for only up to 14 days, necessitating the expense of repeated dosing with Good Manufacturing Process (GMP)-compliant *T. suis* ova[245].

N. americanus currently infects more that 500 million people worldwide, and is thought to be one of the many helminths that was almost ubiquitous in humans until around the 1930s[248]. It therefore has the benefit of a known disease course and a direct theoretical connection to the 'Old Friends' hypothesis; replacing a long-established and recently missed parasite's regulatory influence. As a highly-prevalent helminth infection, *N. americanus* is generally well-tolerated and often asymptomatic. However, infection with this parasite entails a life cycle that involves systemic distribution within the host: penetration of intact human skin followed by migration to the lungs, ascension of the bronchi and trachea, followed by swallowing and eventual establishment of chronic infection with maturation in the small intestine[249]. High worm burdens are associated with iron-deficiency anaemia secondary to intestinal blood loss[250] and other, rarer, complications including anorexia, hypoalbuminaemia and reactive pneumonitis[251]. A number of pilot studies have reported no adverse events from infection with low doses of *N. americanus*

larvae[252,253], but it is not yet known whether the resultant low-grade infections will be sufficient to achieve clinically-relevant immunomodulation.

1.2.6 Clinical Trials of Therapeutic Helminth Infection in Autoimmunity

A total of 25 clinical trials of therapeutic infection with *T. suis* or *N. americanus* are underway or have been completed (recently reviewed by Fleming and Weinstock[239]). Of the trials completed so far, emerging data are reassuring at least in terms of safety, with no reported serious adverse events to date[239]. Whilst all completed trials have so far used relatively small infective doses, and follow-up periods have been relatively short, some reassurance can also be garnered from the wealth of available long-term epidemiological and observational data from natural infections. This contrasts with the long-term complete unknown of, for example, infusion of Treg following *ex-vivo* expansion.

Interestingly, early studies of therapeutic *T. suis* infection for inflammatory bowel disease (IBD) reported that infection was well-tolerated with no notable adverse effects[254-256], but were contradicted by later studies of *T. suis* therapy for allergic rhinitis[257] and MS[258], in which 50% of participants reported gastrointestinal side effects such as abdominal pain and nausea. Side effects were reported as mild and therefore perhaps unnoticed by patients suffering from longstanding IBD. However, this finding does have relevance to the viability of therapeutic infection as a mainstream clinical option, particularly as the infective dosages used were smaller than that of most natural infections, upon which the previously described encouraging observational data is based.

Results from the few interventional clinical studies that have been completed are less encouraging with regard to the efficacy of helminth infection as a therapeutic agent. Two double-blind RCTs investigating treatment of IBD have been reported on. Summers et al[256] recruited 54 patients with active ulcerative colitis and randomised them to fortnightly treatment with 2,500 T. suis ova or placebo for 12 weeks,

followed by a median follow-up period of a further 12 weeks. No significant difference in the rate of clinical remission was observed between treatment groups, but investigators did report a marginally significant reduction in disease severity, as determined by reductions in the Ulcerative Colitis Disease Activity Index (comprised of scoring for frequency of diarrhoea, blood in stool, mucosal appearance, and overall assessment of clinical response) – a response rate of 43% for ova therapy vs. 17% for placebo, p = 0.04, as determined by a 2-sided Fisher's exact test). Sandborn et al conducted the second double-blind RCT in 2013[259], randomising 36 patients with established Crohn's disease to placebo or a variable dose of *T. suis* ova (400 – 7,500) with 6 months of follow-up. The primary outcome was safety and tolerability; no dose-dependent adverse effects were reported, but nor was any clinical improvement seen. A subsequent review by the Cochrane Collaboration dismissed clinical improvement in Summers *et al's* study[256] as insignificant, based on comparison of the risk ratios for patients treated with ova compared to placebo (RR: 2.6, 95% CI 0.97 to 6.95), and concluded that *"there is insufficient evidence to allow any firm conclusions regarding the efficacy and safety of helminths used to treat patients with IBD.* [260]*"*

Preliminary studies investigating the role of helminth therapy in combating MS benefit from the ability to objectively monitor disease activity by measuring new and enlarging inflammatory lesions of the brain and spinal cord with MRI scanning[261]. As MRI is a non-ionising imaging modality, serial scans can be performed for each study subject before, during and after a period of treatment, and can easily be assessed objectively by a radiologist blinded to the treatment conditions. The first clinical study, 'Helminth-induced immunomodulation therapy' (HINT 1) [258], was powered only to assess short-term safety concerns, with recruitment of 5 patients with newly diagnosed MS. Study subjects were given 2,500 *T. suis* ova for 3 months; no adverse events were reported and MRI findings were encouraging, but not statistically significant. A follow-up phase I study (HINT 2, NCT00645749) with 15 patients undergoing 10 months of treatment with *T. suis* has since been conducted but not yet reported any results. Two further studies have also commenced, but similarly not yet reported any interim results. '*Trichuris suis* ova in relapsing-

remitting multiple sclerosis and clinically isolated syndrome' (TRIOMS)[262] is anticipating recruitment of 50 subjects for randomisation of treatment with *T. suis* ova or placebo over 12 months, and 'Worms for Immune Regulation in Multiple Sclerosis' (WIRMS) is anticipating recruitment of 72 subjects for randomisation to treatment with *N. americanus* or placebo over 9 months (NCT01470521).

The therapeutic potential of *Necator americanus* has so far been investigated with clinical trials of live infection in asthma[263] and coeliac disease[264]. Both studies were limited by small number of subjects and difficulty in blinding subjects and investigators to treatment vs. placebo as a result of characteristic abdominal discomfort shortly after infection and pruritus at the site of cutaneous inoculation. As assessed by primary outcome measures, significant clinical improvement in asthma or coeliac disease has not been demonstrated with *N. americanus* infection. However, chronic low-grade infection was successfully established and, despite some initial discomfort, 23 out of 26 infected subjects declined anti-helminthic treatment at the end of the study periods.

A number of possible explanations have been put forward as to why preliminary results from prospective randomised controlled trials have not lived up to expectations based on observational studies. First, and most plausibly, trial design: the small number of preliminary trials that have been performed to date have all been of limited power, particularly given the number of potentially crucial variables that exist (e.g. dosage, timing of infection, severity or stage of the target immunological condition) and, particularly in IBD, the often subjective outcome measures can result in a considerable placebo effect. Secondly, choice of therapeutic helminth: as discussed above, *T. suis* and *N. americanus* were (appropriately) chosen based on their safety characteristics, but it is possible that the least pathogenic helminths are also the least effective at subverting the host immune response. Whilst harmful helminths such as *Schistosoma mansoni* would be clearly unsuitable as live therapeutic agents, further investigation of the immunomodulatory mechanisms at their disposal may yield more effective novel pharmaceutical agents. Finally, Rook *et al*[265] suggest that early childhood exposure to helminth infection may lead to important epigenetic changes that 'pre-condition' a regulatory response upon

subsequent exposure, explaining that the landmark observational studies in MS by Correale *et al*[207,208] were performed in Argentina, where helminth infection is endemic[265]. This may be true and, if so, provides little room for optimism on future clinical translation.

No clinical trials of helminth-derived immunomodulators have yet been performed, largely as a result of the considerable investment and negotiation of necessary regulatory hurdles required of the pharmaceutical industry[266]. As previously discussed, treatment with novel medications based on helminth-derived immunomodulatory products offer numerous advantages over therapeutic live infection; as further results emerge from clinical trials currently underway, the level of evidence required for the necessary pharmaceutical investment might be reached in the near future.

1.3 Helminths and Organ Transplantation

1.3.1 Background to helminth-mediated allograft protection

The prospect of utilising helminths as a therapeutic option in transplantation is one that has received much less attention than in the field of autoimmunity. A number of barriers to investigation exist, not least that transplants are generally performed in high-resource countries with a low prevalence of helminth infection and patients receiving transplants are uniformly prescribed powerful immunosuppressant agents that would likely mask any beneficial effect of infection. Replication of the observational studies investigating helminths and autoimmunity is therefore all but impossible, however unique opportunities also exist: the pathophysiology of allograft rejection is very well described and, in contrast to most autoimmune conditions, the precise timing of onset of 'disease' is also known in advance. A total of 10 studies investigating helminth-derived allograft protection (Table 1.2) have been performed since 1972; these are summarised below with details for each parasite system given in the succeeding sections.

Authors	Parasite	Allograft Model	Fold Graft prolongation	p Value
Hepiretihan *et al.* (2012)[267]	*Echinococcus multilocularis*	Rat heart	2.04	<0.05
Li *et al.* (2011)[268]	*Echinococcus multilocularis*	Rat liver	1.57	<0.05
Liwski *et al.* (2000)[269]	*Nippostrongylus brasiliensis*	Mouse heart	2.80	<0.03
Ledingham *et al.* (1996)[270]	*Nippostrongylus brasiliensis*	Rat kidney	3.30	<0.001
	Nippostrongylus NES	Rat kidney	2.22	<0.001
Aboul-Enein *et al.* (1982)[271]	*Schistosoma mansoni*	Human skin	2.21	0.001
Araujo *et al.* (1977)[272]	*Schistosoma mansoni*	Mouse skin	1.5*	<0.001
Svet-Moldavsky *et al.* (1969)[273]	*Trichinella spiralis*	Mouse skin	2.13	Not stated
Faubert and Tanner. (1975)[274]	*Trichinella spiralis*	Mouse skin	1.89	<0.001
	Infected mouse serum	Mouse skin	1.67	Not stated
Chimyshkyan *et al.* (1976)[275]	*Trichinella spiralis*	Mouse skin	2.48	<0.001
Alkarmi *et al.* (1995)[276]	*Trichinella pseudospiralis*	Mouse skin	3.57	Not stated
	Trichinella pseudospiralis extract	Mouse skin	2.0	Not stated
	Trichinella spiralis	Mouse skin	3.57	Not stated
	Trichinella spiralis extract	Mouse skin	2.43	Not stated

Table 1.2 Studies of helminth-mediated immunomodulation in transplantation.

1.3.2 *Echinococcus* tapeworms (cestodes)

The metacestode *Echinococcus multilocularis* (*Em*) is endemic amongst foxes in many parts of Europe and China and humans can be affected as paratenic hosts. Infection usually results in severe disease with a clinical course resembling that of a malignant primary liver tumour (hepatocellularcarcinoma)[277]. Radical surgical resection of liver lesions has been shown to be effective in improving survival and orthotopic liver transplantation is now largely accepted as appropriate treatment for advanced disease[278]. Disease progression caused by the parasite has been found to advance rapidly in the presence of host immunosuppression either as a result of medication[279] or Human Immunodeficiency Virus infection[280]. This finding led to guidelines recommending reduced immunosuppression regimens following liver transplantation for *Em*[281] and long-term follow up reported unexpectedly satisfactory tolerance of the allografts[278]. Li *et al* have subsequently corroborated this finding in an experimental rat model of liver transplantation with *Em* infection[268] (Table 1.2). In this study, survival following orthotopic liver transplantation was found to be significantly prolonged for *Em*-infected rats compared to naïve controls (15.5 ± 3.9 days vs. 9.9 ± 2.3 days, $p<0.05$). The *Em*-infected group was also found to have reduced $CD4^+$, $CD8^+$ and $CD28^+$ T cell populations in peripheral blood, raised serum IL-10 levels and reduced histological liver allograft rejection scores, all of which reached statistical significance ($p<0.05$)[268]. More recently, Hepiretihan *et al* have shown that *Em* infection exerts a similar protective effect against rejection of rat heart allografts (16.2 ± 3.2 days vs. 7.9 ± 1.9 days)[267]. This was associated with a reduction in graft-infiltrating $CD8^+$ lymphocytes and a shift towards a Th2 cytokine profile in the serum of peripheral blood. In the clinical setting, eradication of *Em* infection usually proves impossible. It therefore remains as yet unclear as to whether graft protection is afforded by an on-going influence of the parasite, or as the result of a Th2 cytokine environment at the time of alloantigen presentation.

1.3.3 *Nippostrongylus* roundworms (nematodes)

In 1996, Ledingham and colleagues demonstrated marked improvement in the survival of kidney allografts in rats infected with the gastrointestinal nematode, *Nippostrongylus brasiliensis* (*Nb*), or inoculated with its secretory products, compared to naïve controls (32 ± 10 days, 21 ± 4.6 days and 9.7 ± 1.2 days, respectively; $p<0.001$)[270]. Representative histological examination five days after the transplant showed a dramatic reduction of graft cellular infiltration in the *Nb*-infected group and this finding was supported quantitatively with flow cytometric analysis of digested allograft single cell suspensions (84% and 81% reduction of $CD8^+$ and $CD4^+$ lymphocytes respectively). Whilst the graft protection afforded by *Nippostrongylus* excretory-secretory products (NES) was less pronounced, the pharmacokinetic profile of the active mediator(s) in NES is unknown and this may therefore be a purely dose-dependent difference.

The same group later showed similar graft protection in a mouse cardiac allograft model[269]. *Nb* infection is known to induce a strong Th2 response in its host[282], leading those authors to hypothesise that polarisation away from Th1-mediated allograft rejection may afford allograft protection. *Nb* usually achieves only a limited infection in rodents - most mouse strains can clear the infection within ten days of inoculation with 3rd stage larvae[282]. The finding that mouse heart allografts can survive for considerably longer than the period of infection[269], presents the exciting therapeutic prospect that graft protection is afforded by T cell 'phenotype switching' at the time of alloantigen presentation rather than a mechanism dependent on persisting parasite infection. ELISA analysis of mixed lymphocyte reactions supports this hypothesis in demonstrating a Th2 cytokine profile (IL-4 and IL-6) in alloreactive lymphocytes from *Nb*-infected mice compared to naïve controls[269,283].

1.3.4 Schistosome flukes (trematodes)

Schistosoma are a genus of blood-borne trematode with a current prevalence of infection estimated at more than 200 million people worldwide[284]. In light of the

very widespread prevalence of schistosomiasis and the diminishing supply of suitable cadaveric donor organs for transplantation, a number of human liver[285,286] and kidney[287] transplants have been performed in patients with clinical schistosomiasis (donor and recipient, donor alone, and recipient alone). No attempts at reducing immunosuppression or analysing differences in rejection rates have as yet been reported. However, one remarkable study has looked at the differential rejection of full-thickness skin grafts in Egyptian patients with established schistosomiasis compared to healthy volunteers. Aboul-Enein and colleagues[271] recruited 19 patients with advanced *Schistosoma mansoni* infection and 16 parasite-free volunteer controls. 2.5cm-diameter full-thickness skin grafts were applied to the volar forearm. Two grafts were performed for each patient: one ABO-matched allograft from a non-infected donor and one autograft control. Grafts were assessed daily for signs for rejection and rejection was then confirmed histologically. The control group rejected their allografts after a mean of 10.06 ± 3.21 days. Of the *Schistosoma*-infected patients, in 16 cases rejection occurred after a mean of 22.25 ± 6.46 days. The remaining three infected patients showed no signs of rejection 60 days after the grafting procedure. Notably, the HLA status of donors and recipients was unknown in this study and therefore the three cases of long-term graft tolerance may well be the result of coincidental HLA matching. In spite of this significant caveat, the difference in rejection times between the two groups was highly significant ($p<0.001$) and therefore unlikely to be the result of differences in HLA matching alone.

Allograft protection with *Schistosoma* infection has previously been shown in a murine experimental model. In 1977, Araujo *et al* found a highly significant difference in the rejection of fully-allogeneic skin grafts in *Schistosoma mansoni*-infected vs naïve recipient mice[272]. No difference was found after thirty days of infection, but for grafts performed after sixty days of infection, infected recipients tolerated their grafts for an average of 50% longer than naïve controls. A strongly positive correlation between graft survival and the number of live parasites remaining in the recipient was also seen ($r=0.096$).

1.3.5 *Trichinella spiralis*

Finally, murine experimental models of other helminth species have also demonstrated enhanced tolerance of skin allografts. *Trichinella spiralis* is a small nematode that encysts in mammalian muscle and can affect humans who consume infected meat. Suppression of skin allograft rejection in mice infested with *Trichinella* was first described by Svet-Moldavsky *et al* in 1969[273] and subsequently confirmed by Faubert[274] (1975) and Chimyshkyan[275] (1976). In 1995, Alkarmi *et al* performed fully-allogeneic skin grafts (C57BL/6 to BALBc recipients and *vice versa*) on multiple groups of mice at varying time points following infection[276]. Graft protection was found to be critically dependent on the timing of skin transplantation in relation to initial infection and a maximum effect of 3.5-fold prolongation of graft survival was found when the transplants were performed 3 days after initial infection. Repeated intraperitoneal injection of parasite secretions (culture supernatants) replicated the effect of active infection in a dose-dependent fashion with an observed maximum 2-fold prolongation in graft survival[276].

1.3.6 *Heligmosomoides polygyrus*

Heligmosomoides polygyrus is a gastrointestinal nematode with powerful immunomodulatory capabilities, notably including induction of $Foxp3^+$ Treg[233,288]. To date, no studies have investigated the potential role for *H. polygyrus* in preventing rejection of solid organ allografts. However, a recently published study by Li *et al* presents interesting inferable results from experimental murine haematopoietic stem cell transplantation (HSCT)[289].

HSCT is an often lifesaving procedure that can be used to treat a variety of haematological malignancies. Traditionally, treatment would begin with a period of chemotherapy or radiation to almost completely ablate the patient's bone marrow prior to infusion of donor cells. This strategy carries considerable risk, particularly if problems with donor cell engraftment arise. As a result, many protocols are now modified with a less aggressive ablative strategy, relying on the healthy immune cells

of the donor population to kill residual neoplastic cells of the recipient (graft vs. tumour effect)[290]. However, if a degree of allogeneic mismatch exists between donor and recipient, there is a risk of the donor cell population recognising 'self' antigens of the recipients as allogeneic and attacking them. This results in 'graft vs host disease' (GvHD), which can be acute or chronic, involving multi-organ pathology (e.g. colitis, pneumonitis, dermatitis) and carry a considerable risk of mortality. Acute GvHD can be treated with aggressive immunosuppression in a similar way to acute rejection of solid organ transplants, but this encompasses all of the adverse effects previously discussed and, crucially, can significantly compromise the graft vs. tumour effect, resulting in relapse of the original malignancy and a higher risk of mortality[291].

Li *et al* performed fully-allogeneic (C57BL/6 to BALB/c) murine HSCT with recipient animals receiving either no treatment, or infection with 150 *H. polygyrus* 3rd stage larvae by oral gavage 3 weeks in advance[289]. From 6 days after HSCT, the uninfected mice appeared visibly debilitated in contrast to the normal appearance of infected animals and went on to suffer a considerably higher mortality from GvHD (100% vs. 40%, $p<0.0001$ by Kaplan Meier log-rank analysis). Blinded histological scoring of lung and colon specimens at day six post HSCT (from a separate experiment) confirmed severe inflammation consistent with GVHD in the uninfected animals and normal mucosal appearances of those with *H. polygyrus* infection. Further analysis revealed significant expansion of Foxp3^{+} Treg with *H. polygyrus* infection and that *H. polygyrus*-mediated protection from disease was TGF-β-dependent – HSCT with Type II TGF-β Receptor dominant negative donor cells obliterated the protective effect of infection. Finally, immunocompetence in controlling neoplastic cells was assessed by administering luciferase-expressing A20 leukaemia/lymphoma cells. Control mice that received T cell-depleted HSCT died rapidly with an overwhelming tumour burden, whereas no tumour development was observed in both *H. polygyrus*-infected and uninfected mice, suggesting that *H. polygyrus* does not compromise the graft vs. tumour effect.

1.3.7 Mechanisms of allograft protection

It is now clear that helminths act via multiple distinct and synergistic pathways to down-regulate host immunity. Expansion of Treg populations in response to helminth infections such as *Heligmosomoides polygyrus*[288] and *Schistosoma mansoni*[236] is one well-recognised mechanism, but the same parasites can also engender immunosuppressive activity in B cell populations, as well as modified dendritic cell and macrophage populations[292].

In this context, it is possible that therapeutic extension of graft survival would also require more than one particular immunomodulatory pathway. With respect to Treg expansion, exogenous IL-2:anti-IL-2 antibody complex is a potent short-term stimulant of Treg populations which can effect long-term tolerance of allogeneic islet grafts in the absence of immunosuppression[293]. However, multiple attempts to achieve similar tolerance of allogeneic skin grafts (BALB/c to C57BL/6) have failed[294]. It is well known in the experimental and clinical setting that tolerance of skin allografts presents a particular challenge (compared to the solid organ transplants of heart, liver or kidney). Important factors to overcome are likely to be the large proportion of resident dendritic cells in skin[294] and more potent Toll-like receptor stimulation by colonising microbes[295]. Failure of IL-2:anti-IL-2 complexes to achieve the same level of protection of fully allogeneic skin grafts against rejection[294] that is seen in *Schistosoma* infection[271] strongly suggests that Treg-independent mechanisms also play a critical role in helminth-derived allograft protection.

1.3.8 Translational feasibility

Enhanced allograft tolerance with helminth infection has now been demonstrated in multiple species across multiple organ allograft models (mouse heart and skin; rat heart, liver and kidney). These experimental data are consistent with historical results of skin grafting in established human schistosomiasis, and supported by more recent anecdotal suggestions of reduced immunosuppression requirement following

liver transplantation for human *Echinococcus* infection[278]. Thus, the possibility can now be entertained of including specific live (non-pathogenic) helminth infection, or defined products from immunoregulatory helminths, in future transplantation protocols. Ongoing trials of live helminth therapy in autoimmunity are keenly awaited as potential pathfinding studies for translation of this concept to the clinic.

1.4 Secreted Immunomodulatory Products

Identification and reproduction of individual helminth-secreted immunomodulatory molecules as potential novel therapeutic agents presents several advantages over live larval therapy. These include consistent pharmacokinetics, scope for pharmacological modification and optimisation (reducing immunogenicity of large molecules, for example), improved public acceptability and a lower cost barrier to large-scale production as a routine clinical therapy. The transcriptomes of several helminths have now been analysed in detail in the search for key immunomodulatory mediators, including: *Heligmosomoides polygyrus*[296,297], *Necator americanus*[245], *Nippostrongylus brasiliensis*[298] and *Trichuris suis*[245,299]. However, proteomic analysis of (for example) H. polygyrus has revealed a daunting number of candidates (>300 ES molecules), many of which show no homology to any known protein[296].

Interestingly, individual helminth species appear to have evolved independently and therefore achieve immunomodulatory capabilities through numerous and often distinct mechanisms[300]. This reinforces the concept that each parasite is very specifically adapted to particular immunological mechanisms and, by extension, their secreted products are likely to be most suited as therapeutic candidates to some specific disease processes more than others. Similarly, products that have evolved to optimally modulate immunity in one host species may not be effective in another.

Live infection with *Schistosoma mansoni* has been shown to ameliorate allograft rejection in both mice[272] and humans[271] (section 1.3.4), but infection is clearly associated with considerable morbidity[301], such that therapeutic infection is not an attractive or feasible prospect. The effects of *Schistosoma* soluble egg antigen (SEA)

and its components have been studied widely, however, and may offer therapeutic opportunities that are translationally viable. *In vivo*, SEA has been shown to be effective in treating EAE[229], murine T cell transfer-mediated colitis[302] and type I diabetes in non-obese diabetic (NOD) mice[236].

SEA is known to act on DCs to induce strong Th2 responses in murine[303] and human cells[304], and this effect has subsequently been ascribed to the action of a glycoprotein component, Omega-1[305,306]. SEA is also capable of suppressing $CD4^+$ T cell proliferation and inducing Foxp3 expression indirectly[236]. Whist DCs are necessary for SEA-driven Foxp3 induction, an increase in the expression of TGF-β latency associated peptide on the surface of $CD4^+$ cells, suggests that SEA also induces enhanced secretion of TGF-β by activated T cells, further facilitating $Foxp3^+$ Treg differentiation[236]. Omega-1 glycans within SEA bind to the mannose receptor of DCs, which enables internalisation and interference with mRNA and protein synthesis[307]. This probably accounts for the fact that Omega-1 has the ability to indirectly induce $Foxp3^+$ Treg with high levels of CTLA-4 expression (through alteration of DCs), but does not affect T cell proliferation or T cell TGF-β secretion. The specificity of Omega-1 also suggests that multiple components of SEA promote Treg differentiation through distinct mechanisms[308]. Finally, Omega-1 is unfortunately both immunogenic and hepatotoxic[309], so would require some modification to become therapeutically viable.

Another component of SEA that has been identified is the $Lewis^x$ trisaccharide. Lacto-N-fucopentaose III (LNFPIII), is a glycan with very low cytotoxicity that contains $Lewis^x$ and is also found in human breast milk[310]. LNFPIII has multiple immunomodulatory capabilities including DC-derived Th2 differentiation[311], alternative activation of macrophages[312] and stimulation of IL-10 production from DCs and macrophages[312]. Dhutta *et al* have shown that LNFPIII induces high levels of programmed cell death ligand 1 (PD-L1) expression on the surface of macrophages, DCs and T cells, which indirectly promotes Treg differentiation[313]. With two transplant models (vascularised adult heterotopic cardiac allografts and non-vascularised neonatal cardiac allografts), these authors showed that LNFPIII was

able to significantly prolong allograft survival and that this protective effect was dependent on PD-L1:PD-1 signalling.

LNFPIII has also been shown to ameliorate EAE, but potentially by different means. With an active ($pMOG_{35-55}$) EAE model in C57BL/6 mice, Zhu *et al* administered LNFPIII conjugates or control dextran twice per week by IP injection and observed a significant reduction in disease severity as assessed by clinical scoring and the presence of inflammatory foci in the CNS upon histological analysis at day 20 post immunisation[228]. Immunohistochemistry revealed a reduction in CNS-infiltrating $CD4^+$, $F4/80^+$ and $CD11c^+$ cells in LNFPIII-treated animals; Foxp3 expression was not reported. At day 9 post immunisation, $CD11b^+Ly6C^{hi}Ly6G^-$ monocytes were isolated from the spleen and LNFPIII treatment was found to result in significantly elevated expression of nitric oxide synthase 2 (Nos2) mRNA. When the same monocyte populations were then cultured *ex vivo* with MOG-specific transgenic $CD4^+$ (2D2) cells and MOG-pulsed APCs, T cell proliferation was suppressed by monocytes from LNFPIII-treated animals and unaffected by the dextran-treated controls. This suppression was completely reversed by L-NIL (a Nos2 inhibitor), confirming that the observed suppressive activity of monocytes was mediated by nitric oxide.

Of all helminth secreted products, *H. polygyrus* ES (HES) is perhaps the most extensively characterised and has been found to contain numerous immunomodulatory components with distinct mechanisms of action[288]. However, in relation to allograft tolerance, perhaps the most significant finding is the ability of HES to induce $Foxp3^+$ Treg differentiation independently of DCs (in common with the secreted products of *Teladorsagia circumcincta*). Grainger *et al* first showed that HES is able to induce Foxp3 expression in isolated $CD4^+Foxp3(GFP)^-$ T cells *in vitro* with stimulation from Concanavalin A or plate-bound CD3/CD28[233]. HES also suppressed proliferation of $CD4^+Foxp3(GFP)^-$ T cells and promoted IL-17 expression from naïve $CD4^+$ cells when co-cultured with IL-6. This led to further investigation with a TGF-β reporter cell line ($TGF\text{-}\beta^{-/-}$ fibroblasts transfected with a TGF-β-responsive alkaline phosphatase reporter, described in detail in Figure 5-1), which confirmed TGF-β activity within HES that could be completely ablated with a

type I TGF-β receptor kinase inhibitor, but was unaffected by a pan-vertebrate anti-TGF-β blocking antibody. Thus, HES contains a TGF-β mimic that induces Foxp3 through ligation of the TGF-β receptor complex, but is sufficiently structurally dissimilar to mammalian TGF-β as to be unaffected by a neutralising antibody. This work marked the beginning of a search for the TGF-β mimic within HES and its mechanism of action, some results of which are presented in chapter 5.

Identification of key immunomodulators and therapeutic candidates within ES products engenders numerous challenges including specificity of action and the possibility of combinations of molecules being required for effective replication of the immunoregulation achieved by live infection[224,314]. However, in comparison to the coincidental discovery of immunosuppressive properties with currently used medications[315], ES products provide a rich source of candidate immunomodulators with the benefit of millennia of evolutionary refinement and a favourable profile of adverse effects in the billions of people that have been exposed to them over long periods worldwide.

1.5 TGF-β Signalling

The TGF-β superfamily of cytokines is comprised of more than 30 distinct molecules including three isoforms of TGF-β, Bone Morphogenic Proteins (BMPs), activins, inhibins, nodal and growth differentiation factors[316]. This group of ligands can initiate pleiotropic effects in a vast array of biological processes including embryogenesis, immunity, angiogenesis and wound healing[317]. In light of the multiplicity of consequences that can result from TGF-β signalling, it is perhaps unsurprising that a complex signalling arrangement with several layers of regulation exists[318].

Regulation of TGF-β signalling takes place predominantly in three distinct regions: the extracellular space, the cell membrane and the intracellular region. TGF-β is synthesised as a component of a larger, inactive molecule that incorporates a ‘latency-associated peptide’ (LAP). Prior to secretion from a cell, the LAP-TGF-β

complex binds to a further protein, latent TGF-β binding protein (LTBP). Because TGF-β is secreted in this way, as a component of a biologically inactive compound, processes that liberate the active TGF-β molecule can be as important to immunomodulation as its transcription and synthesis[319]. In the extracellular space, the active TGF-β molecule is also prone to sequestration by 'ligand trap' proteins (including LAP), such that active TGF-β cannot persist for long and cleavage mechanisms are afforded greater significance[320].

Cleavage of the active TGF-β molecule can only happen under specific conditions, such as low pH, proteolysis, or the binding of certain membrane-bound proteins[321]. *In vivo*, cell surface receptors including many integrins have the ability to bind LAP-TGF-β and release active TGF-β[322]. This mechanism is of considerable importance – αv integrin null mice develop similar embryological aberrations to TGF-$\beta^{-/-}$ animals[323]. Indeed, functioning integrin-TGF-β interaction may be a key determinant of some tolerogenic cell phenotypes – the ability of DCs from $\alpha v\beta 8^{-/-}$ mice to induce Treg is considerably impaired and these mice consequently develop spontaneous colitis in normal environmental conditions[324].

Activated TGF-β signals as a homodimer via the union of two type I TGF-β receptors (TβRI, also known as ALK5) and two type II TGF-β receptors (TβRII). In humans, 5 variants of the type I receptor and 7 variants of the type II receptor have been identified, in contrast to 29 potential ligands[325]. The affinity of each ligand for individual receptors varies, but most ligands are also able to bind multiple heteromeric combinations of type I and type II receptors, resulting in different downstream effects[325]. Upon binding of the TGF-β ligand, the constitutively active type II receptor is brought into close proximity of the type I receptor, thereby enabling phosphorylation of the TβRI intracellular 'GS' domain and initiating the Smad signalling cascade[326] (Figure 1-6). Smads are intracellular proteins that mediate signalling from TGF-β receptors to the nucleus; 8 Smad proteins have been identified in vertebrates and they are sub-categorised according to their function: receptor-activated Smads (R-Smads), common Smads (Co-Smads) and inhibitory Smads (i-Smads)[326]. When TGF-β binds and activates the TGF-β receptor complex, the intracellular GS domain of TβRI phosphorylates Smad2 and Smad3 (R-Smads),

which then form a complex with Smad4 (Co-Smad) and enter the nucleus to initiate gene transcription[116]. This process can be regulated intracellularly by Smad7, an inhibitory Smad that can bind TβRI, prevent further signal transduction and then stimulate proteolytic degradation of the receptor[326]. At the level of the cell membrane, another regulatory mechanism comes into play in the form of 'decoy' receptors such as BAMBI (BMP and activin membrane-bound inhibitor), a transmembrane protein that is structurally very similar to TβRI, but lacks an intracellular GS domain[327]. BAMBI can therefore form a dimer with TβRII and bind TGF-β without initiating any Smad signalling and thereby reduce the number of TβRIIs available to bind other TGF-β molecules.

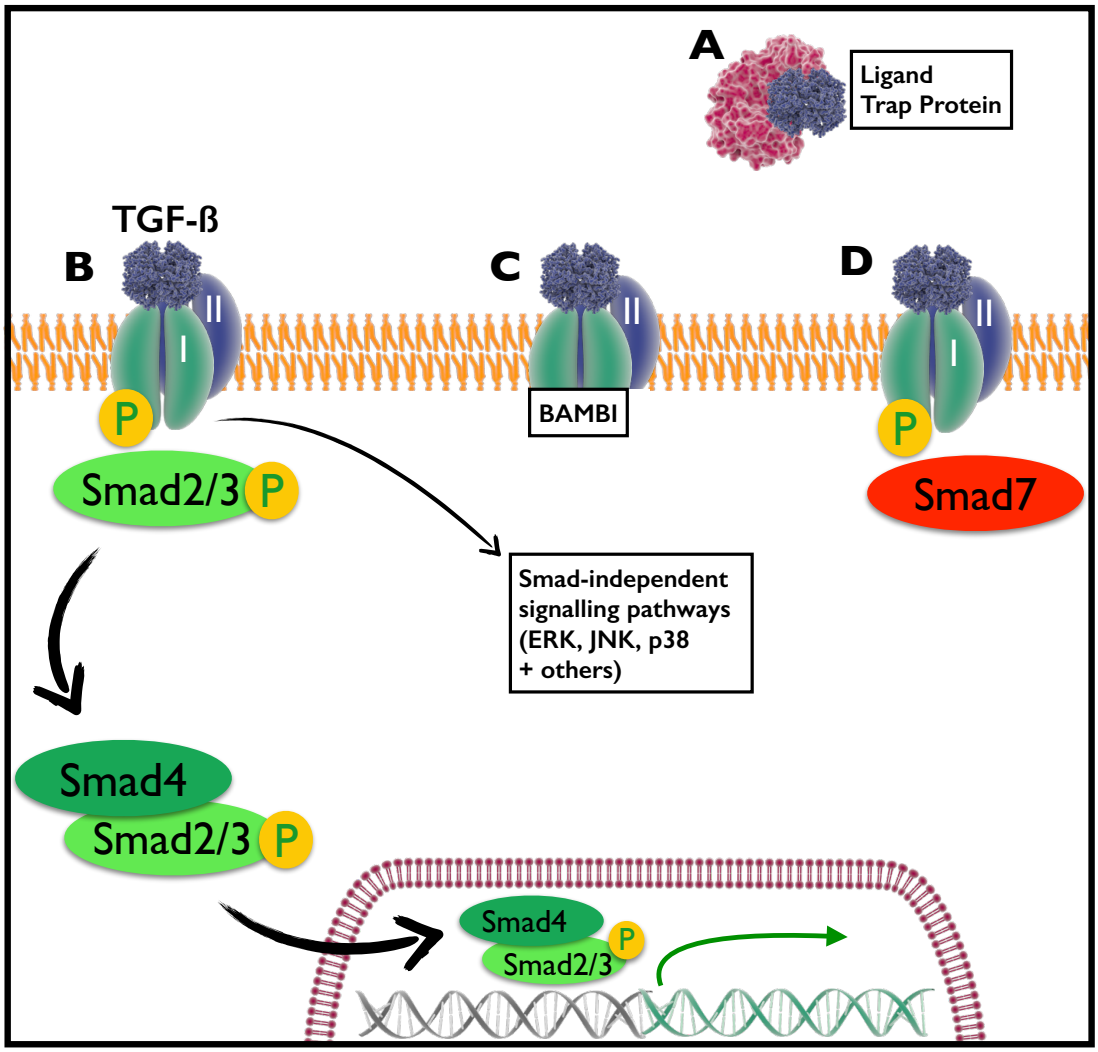


Figure 1-7 TGF-β Signalling and Regulation. (A) Active TGF-β is prevented from binding to receptors due to incorporation with 'ligand trap proteins' such as latency-associated peptide (LAP). (B) Binding of TGF-β to the Type II TGF-β receptor leads to phosphorylation of the Type I TGF-β receptor intracellular domain and activation of the Smad signalling pathway, in addition to Smad-independent signalling including MAPK pathways. (C) Decoy receptors such as BAMBI bind TGF-β but prevent downstream signalling. (D) Smad7, an inhibitory Smad, binds to the phosphorylated Type I TGF-β receptor and prevents downstream signalling.

The Smad cascade is the 'canonical' signalling pathway for TGF-β and is essential for TGF-β-driven immunoregulation and Treg/Th cellular differentiation[328]. However, TGF-β is also able to activate a number of Smad-independent signalling pathways including mitogen-activated protein kinases (MAPKs). Of these, ERK

phosphorylation is an important event in the process of epithelial to mesenchymal transition (EMT), which is necessary in embryological development, but can contribute to pathological fibrosis[329]. EMT also promotes the ability of tumour cells to metastasize and therefore small molecule inhibitors are under investigation in this context[330], but could prove to be useful for the prevention of TGF-β-related fibrosis in transplantation as well.

In vitro stimulation of naïve $CD4^+$ T cells in the presence of TGF-β reliably leads to the induction of $Foxp3^+$ Treg that can suppress effector T cell activation and proliferation[331], and prolong allograft survival upon adoptive transfer into recipient animals[332]. The level of Foxp3 expression by Treg correlates with functional suppressive capacity[333]; stability of Foxp3 expression is essential for maintenance of a regulatory phenotype[334]. TGF-β-dependent induction of Treg also occurs *in vivo* and techniques that exploit this mechanism to induce tolerance (such as low dose antigen therapy[335]) appear to generate Treg with more stable expression of Foxp3 than those generated *in vitro*[336]. This suggests that additional (unknown) stabilising factors or conditions are present in the *in vivo* setting and might provide encouragement for *in vivo* Treg induction techniques over *ex vivo* expansion and reinfusion of isogeneic cells. Additionally, TGF-β is capable of inducing tolerance through Foxp3-independent mechanisms, such as upregulation of CD73[337], an ectoenzyme that acts to increase the local extracellular concentration of adenosine (as discussed in section 1.1.4.3).

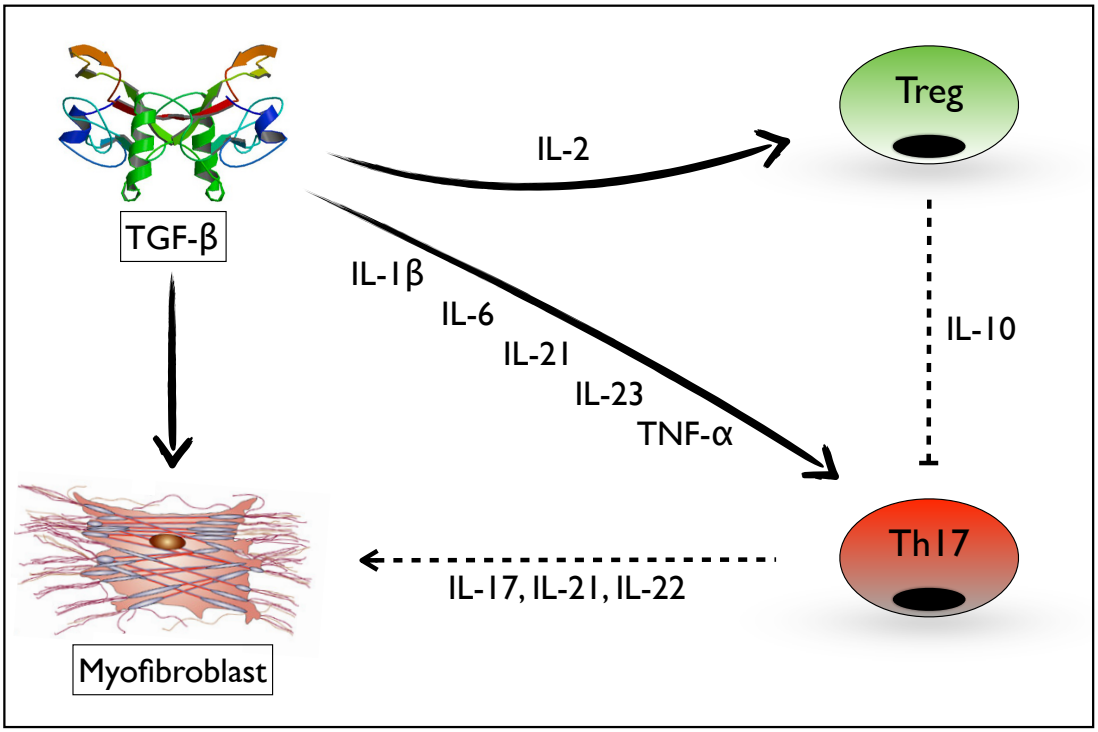


Figure 1-8 TGF-β and Cellular Differentiation (adapted from Hegner *et al*[338]).

As previously discussed, TGF-β does have the potential to play janiform roles in the context of transplantation. Whilst TGF-β-induced immunoregulation is clearly beneficial to an allograft, detrimental effects can also arise through generation of Th17 effector cells and/or interstitial fibrosis as a result of increased myofibroblast differentiation[338] (Figure 1-7). IL-1β, IL-6 and TLR ligands can all promote Th17 differentiation[338,339] (Figure 1-8), in addition to the frequently used immunosuppressant agents, ciclosporin and tacrolimus (through blockade of IL-2)[340]. This clearly cautions against the use of enhanced TGF-β activity as a therapeutic strategy. However, there is some evidence that Th17 cells can be generated in the absence of TGF-β[341] and that without the influence of TGF-β, these Th17 cells are more pathogenic due to enhanced IL-23 receptor expression. Additionally, high concentrations of TGF-β appear to promote Treg differentiation over a Th17 phenotype[342].

Combination therapy with synergistic 'Treg permissive' agents may allow some control over downstream cellular differentiation. Of these, rapamycin (sirolimus) can act synergistically with TGF-β to induce Foxp3 expression and Treg differentiation over Th17 effector cells[343] and retinoic acid (RA) can effectively minimise the ability of inflammatory cytokines and co-stimulation to impair TGF-β-induced Foxp3 expression[344,345]. Whilst the non-linear signalling and pleiotropic effects of TGF-β limit the therapeutic potential of the native molecule, it is now clear that helminths have evolved to exploit this pathway very effectively. Identification of how this is achieved could lead to novel therapeutic agents that are more efficacious and less harmful than currently available options in an area of considerable clinical need.

1.6 Thesis objectives

The aims of this thesis are:

1. To establish if *Heligmosomoides polygyrus* infection suppresses allograft rejection and autoimmune disease.
2. To investigate if secreted products from *Heligmosomoides polygyrus* replicates this suppressive effect.
3. To investigate molecular and mechanistic pathways through which suppression might be achieved.

2 Materials and Methods

2.1 General Reagents

All reagents were acquired from Sigma unless otherwise stated.

2.1.1 Complete RPMI

RPMI medium containing HEPES (Gibco), supplemented with 2 mM L- glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco), 10% heat-inactivated foetal calf serum (FCS) (Gibco), and 50 nM 2- mercaptoethanol (Gibco).

2.1.2 ELISA carbonate buffer

1 M solutions of Na_2CO_3 (Sigma) and Na_2HCO_3 (Sigma) were first formed in distilled water. 0.06 M carbonate buffer was then created with the addition of 45.3 ml (1 M) Na2CO3 and 18.2ml (1 M) Na^2HCO_3 to 936.5 ml of distilled water; followed by adjustment of the final solution to pH 9.6.

2.1.3 FACS buffer

PBS was supplemented with 0.5% heat-inactivated FCS (Gibco) and 0.05% sodium azide (Sigma).

2.1.4 MACS buffer

Hanks' Balanced Salt Solution (HBSS, Gibco) supplemented with 2 % heat-inactivated FCS and 2 mM EDTA.

2.1.5 *H. polygyrus* media

RPMI medium, containing HEPES (Gibco), was supplemented with 1% glucose, 2mM L-glutamine, 5 U/ml penicillin, 5 μg/ml streptomycin and 1 μg/ml gentamicin.

2.2 Animals

Inbred C57BL/6 (CD45.1 and CD45.2), BALB/c and CBA mice used for experiments were all aged 6-12 weeks old and bred in-house or purchased from Harlan Laboratories. Transgenic strains (OT-II, DO11.10, RAG1$^{-/-}$, IL-4Rα$^{-/-}$, Foxp3-GFP) were all bred in house. Animals were housed in individually ventilated cages for the duration of experiments. All animal experiments were performed under a Project Licence granted by the UK Home Office and approved by the University of Edinburgh Veterinary Services.

2.3 *Heligmosomoides polygyrus* Life Cycle

2.3.1 Propagation and maintenance of *H. polygyrus*

Day-to-day maintenance of the *H. polygyrus* life cycle was performed by Elaine Robertson and Yvonne Harcus. A more comprehensive protocol (and accompanying video) has been recently published by the Maizels lab[346] and is included in appendix A.

For experimental use, *H. polygyrus* L3 larvae can be safely stored at 4˚C for up to six months. To maintain the life cycle, CBAxC57BL/6 F1 mice were bred and maintained for their ability to withstand a high parasite burden in the absence of morbidity. L3 larvae were prepared by washing three times in dH_2O, counting and resuspending at 2000 larvae / ml. Male F1 mice were then infected with 400 L3 larvae in 200 µl dH_2O by oral gavage. 14 days later mice were culled and intestines removed for adult harvest (see section 2.3.2), together with collection of faeces for larval culture. Faecal samples were mixed with (washed) granulated charcoal (Merck) to achieve a damp paste that was then thinly applied to moist filter paper and placed in a petri dish inside a damp box (in the dark at 37˚C) for 12-14 days. Larvae were collected by washing the filter paper with dH_2O and then stored at 4˚C; collection can take place from day 7 onwards, and was performed on at least two

occasions for each cycle. Following a period of storage, larvae were washed again with dH_2O three times prior to infection by oral gavage (200 L3 for inbred strains).

2.3.2 Isolation of adult *H. polygyrus* worms

F1 mice used to maintain the *H. polygyrus* life cycle were culled 14 days after infection. Post-mortem, the abdomen was prepared with 70% ethanol and the most proximal 20 cm of small intestine was excised (most adult worms are found in the duodenum). The small intestine was then placed in a (100 mm diameter) petri dish with HBSS (warmed to 37˚C) and opened longitudinally to reveal the intra-luminal worms. The worms are best removed by gently scraping the small intestine between two glass slides; following removal of the worms, the gut wall was discarded.

Adult worms were isolated from extraneous debris with a Baermann apparatus. Worms were placed into small muslin bags, which were stapled closed and fixed to a glass funnel as shown (figure 2-1). Once the muslin bags had been filled with worms from approximately 8 mice, the funnel was filled with HBSS and placed in a 37˚C incubator for 2 hours, with intermittent gentle agitation to dislodge any obstructing debris.

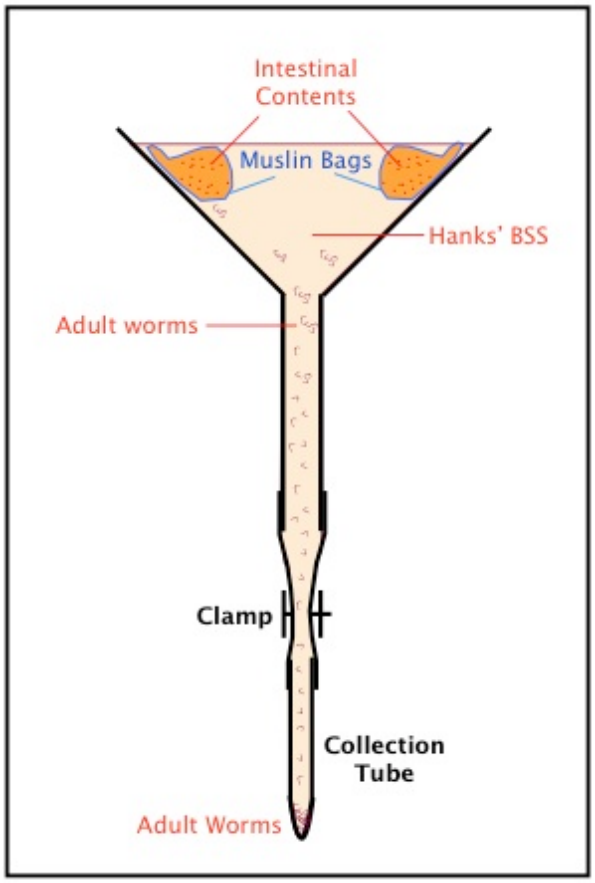


Figure 2-1 Setup of Baermann Apparatus for collection of adult *H. polygyrus.*

After two hours, adult worms had slowly migrated to the bottom of the collection tube, which was then carefully detached, allowing the worms to be transferred to a 50 ml Falcon tube and washed six times with 40 ml of HBSS. Moving to a laminar flow hood, the worms were then washed another six times in sterile HBSS supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin. Adult worms were counted in a volume of 20 µl, with a typical yield of 50% the number of L3 larvae used at the time of inoculation (figure 2-1).

2.3.3 Culture and preparation of HES

In preparation for culture of adult worms to produce HES, freshly isolated worms were incubated for 20 minutes in 10 ml of RPMI (Gibco) supplemented with 10 µg/ml gentamycin and then washed a further six times with sterile HBSS (supplemented with 5 U/ml penicillin and 5 µg/ml streptomycin). The worms were then resuspended in *H. polygyrus* media (2.1.5) and transferred into vented T25 flasks in aliquots of 15 ml containing approximately 1000 worms. The flasks were placed upright in an incubator at 37˚C (5% CO_2) for 3 weeks.

Once the worm culture was established as described above, culture media (containing HES) was removed and replenished with sterile *H. polygrus* media after 24 hours and subsequently at intervals no longer than twice per week. Media collected after the first 24 hours of culture was set aside to minimise the risk of contamination with intestinal contents. Subsequent collections of the culture supernatant were pooled, filter sterilised through 0.2 µm low-protein binding filters and frozen at -20 ˚C for later concentration. At a later date, typically 500 ml of frozen HES-containing culture media (excluding the collection from the first 24 hours of culture) was concentrated over a 3,000 MW cut-off filter under nitrogen pressure in an Amicon ultrafiltration device (Millipore). The amount of HES in each 500 ml batch of culture media was quantified by Bradford assay (Life Technologies, performed as per manufacturer's instructions), with a typical yield of 1.5 mg (figure 2-2b). Finally, a chromogenic Limulus amebocyte Lysate (LAL) assay was performed (according to the manufacturer's instructions) to test the level of LPS contamination. Of 41 batches, the median level of contamination was 86 U/mg of HES; batches with greater than 1000 U LPS per 1 mg of protein were considered unsuitable for future *in vitro* or *in vivo* experimental use (based on an extrapolation of the *in vivo* dose at which LPS begins to suppress allergic airway responses[347]).

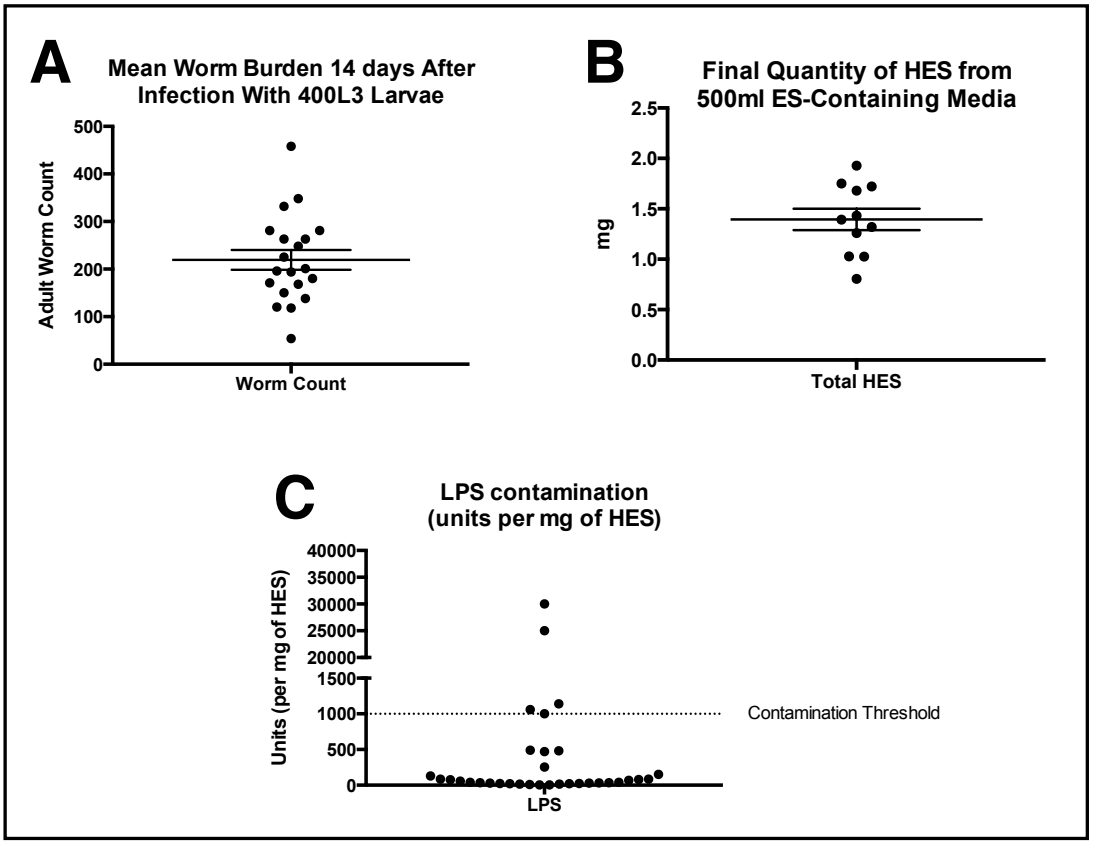


Figure 2-2 (A) Data points shown represent worm burdens from 19 separate rounds of infection of C57BL/6xCBA mice with 400 L3 larvae. (B) Yield of HES protein from 11 different batches derived from approximately 500 ml of culture supernatant. (C) Levels of LPS contamination in 41 batches of HES measured by the Limulus amoebocyte lysate (LAL) assay.

A summary of the complete *H. polygyrus* lifecycle is shown in the schematic, figure 2-3.

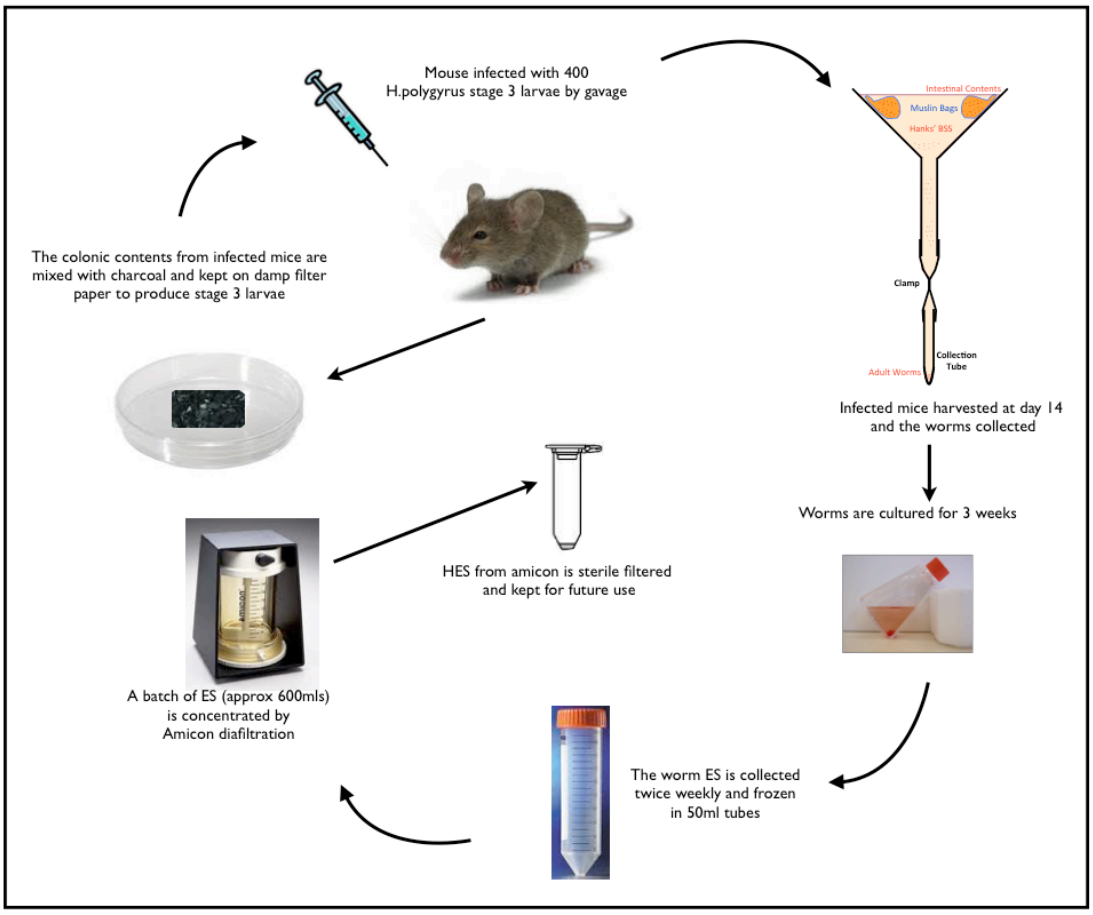


Figure 2-3 Animated Schematic of *H. polygyrus* Life Cycle Summary of key life cycle stages from oral gavage of L3 larvae, through recovery of larvae and adult worms to isolation of HES.

2.4 Full-thickness Skin Grafting

2.4.1 General anaesthesia

General anaesthesia was achieved with either an inhalational or injectable anaesthetic protocol. The body temperature of animals under anaesthesia was maintained with an insulated heat mat at 38°C. For inhalational anaesthesia, oxygen was continuously administered at 2 litres/minute with 4 % isoflurane (Abbott Laboratories Ltd) for induction and 2 % isoflurane for maintenance. Injectable anaesthesia (for a 25 gram mouse) involved 20 µg medetomidine (Domitor®, Vet Quinol Ltd) and 1.5 mg

ketamine (Vetalar®, Zoetis Ltd). A mixture was prepared of 0.5 ml medetomidine at 1 mg/ml, 0.38 ml of ketamine at 100 mg/ml and 4.12 ml water, and each mouse received 200 µl injected intraperitoneally. Reversal of anaesthesia (for a 25 gram mouse) was achieved with 40 µg atipamezole (Antisedan®, Elanco Animal Health Ltd), for which 200 µl of atipamezole at 5mg /ml was mixed with 4.2 ml PBS; 250 µl of this dilution was injected subcutaneously.

For both anaesthetic protocols, buprenorphine (Vetergesic®, Reckitt Benckiser Healthcare Ltd) analgesia was administered on induction of anaesthesia and again 12 hours later. A dose of 0.1 mg/kg was used, (2.5 µg for a 25 gram mouse) by diluting 100 µl of buprenorphine at 0.5 mg/ml with 900 µl of PBS and injecting 50 µl subcutaneously.

Upon adoption of the skin grafting method described below at the University of Edinburgh, inhalational (isoflurane) anaesthesia was used in accordance with local veterinary protocols and regulations. Initially, wide variation in graft survival was seen within experimental groups. Since one donor mouse provides a graft for three recipient animals, it was hypothesised that the variation in survival was due to a wide range of graft ischaemia time for each recipient animal (with inhalational anaesthesia, the skin grafting procedure had to be performed from start to finish on each animal individually. This problem was resolved by changing to injectable anaesthesia, thereby allowing simultaneous anaesthesia for four recipient animals. Preparation of the skin and graft bed was then performed prior to culling the donor animal. Recipient graft beds were temporarily covered in saline-soaked gauze, until donor grafts could be placed almost simultaneously on all of the anaesthetised animals, eliminating variation in graft ischaemia time.

2.4.2 Surgery

Full-thickness skin transplantation was performed initially under the direction and supervision of Dr Andrew Bushell (Transplant Research Immunology Group,

University of Oxford), using a modified technique of that originally described by Billingham and Medawar[348].

Tail skin from donor mice was prepared (immediately post-mortem) by spraying with 70% ethanol, making a circumferential incision around the base of the tail and then extending the incision distally along the ventral midline. The tail skin was then stripped, placed into cold PBS and fashioned into three 1x1 cm squares.

Recipient animals were placed under general anaesthesia prior to shaving the right flank and preparing skin with chlorahexidine solution (Animal Care Ltd). The graft bed was prepared by dissecting skin from the right flank, taking care to preserve underlying subcutaneous adipose tissue (for microvascular blood supply to the graft). Optimally, the skin defect created was slightly larger than the size of the graft (1 mm at each edge), so that the graft remained taught and the risk of seroma formation was minimised. Following placement of the graft onto the graft bed, it was secured in place with methylated flexible collodion (William Ransom & Son Ltd), applied sparingly along the wound edges. Particular attention was paid to avoid excess collodion, which can present an inflammatory insult, to the detriment of the allograft. The grafts were covered with an iodine-impregnated non-adherent dressing (Inadine®, Johnson and Johnson Medical) and then secured in place with autoclave tape (Fisher Scientific), as shown in figure 2-4. The animals were placed in an incubator at 30°C in the immediate post-operative period (for up to 2 hours). Overall operative mortality was less than 2%.

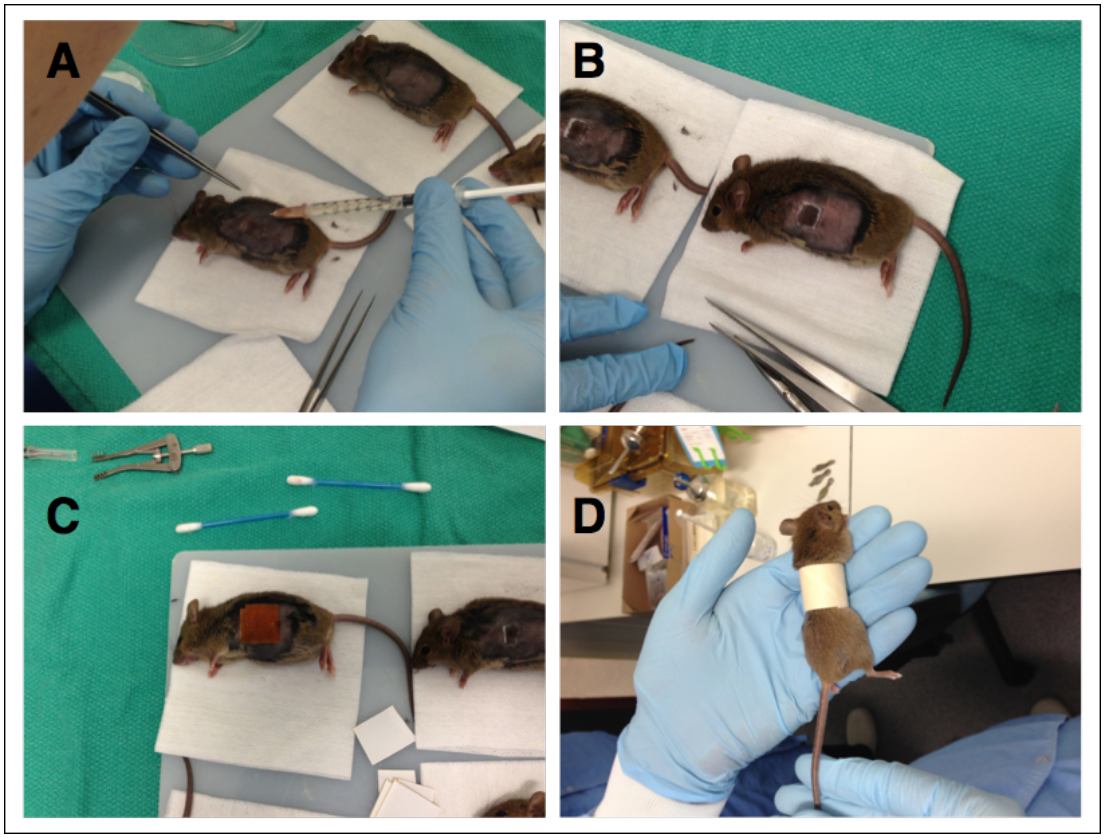


Figure 2-4 Full-thickness skin grafting. Following preparation of the recipient graft bed, the donor graft is placed in position (A), secured with flexible collodion around the wound edge (B), then covered with a non-adherent dressing (C) and finally secured with autoclave tape (D).

Dressings were removed seven days after skin grafting under a brief general anaesthetic (inhalational isoflurane). The grafts were assessed carefully at this point so that damaged grafts (usually from the animal's attempt to remove dressings) could be prospectively excluded from analysis as technical failures. Allografts were monitored on a daily basis following the removal of dressings and rejection was defined as more than 90% necrosis by surface area, or complete dehiscence.

2.5 Experimental Autoimmune Encephalomyelitis (EAE) Models

2.5.1 Wild-type C57BL/6 Model Immunisation

Active EAE induction was achieved according to a protocol described by O'Connor *et al*[349]. Female C57BL/6 mice were immunised with 100 µg $pMOG_{35-55}$ and Complete Freund's Adjuvant containing 50 µg heat-killed *M. tuberculosis*. Emulsification of the immunisation mixture was achieved with a sonicator and a final volume of 100 µl per mouse was administered – 50 µl by subcutaneous injection in each hind limb.

2.5.2 Tg4 Transfer model immunisation

Passive EAE induction was achieved according to the protocol described by McPherson *et al*[350]. One day following adoptive transfer of 10^6 Tg4 antigen-specific $CD4^+$ cells, B10xB6 mice were immunised with 10 µg $Ac1\text{-}9_{(4Y)}$ peptide in emulsion with Complete Freund's Adjuvant containing 30 µg heat-killed *M. tuberculosis*, via a subcutaneous injection of 50 µl into each hind limb.

2.5.3 Pertussis Toxin

Following immunisation with $pMOG_{35-55}$ (C57BL/6) or $Ac1\text{-}9_{(4Y)}$ (B10xB6), 200 ng pertussis toxin (PTX) was administered by intraperitoneal injection in a volume of 500 µl PBS on the same day and again 48 hours later (i.e. day 0 and day 2).

2.5.4 Monitoring and Clinical Scoring

Daily monitoring for features of clinical disease commenced on the sixth day following immunisation. The weight of all mice was recorded on day 0 and animals were culled if they were found to lose 30% or more of their original body weight.

Other humane end-points necessitating euthanasia included a clinical score of '5' or additional descriptor 'E' on two consecutive days.

Classical EAE Scoring				
Clinical Features	**Score**		**Additional Descriptors**	**Annotation**
Healthy	0		Pilo-erection	A
Flaccid tail	1		Reduced activity	B
Impaired righting reflex and/or impaired gait	2		Hunched posture	C
Substantially impaired gait (not dragging limb)	2.5		Lying on side (mobile)	D
Partial hind limb paralysis (dragging limb)	3		Lying on side (immobile)	E
Total hind limb paralysis	4			
Any sign of forelimb paralysis	5			
Moribund or found dead	6			

Table 2-1 Classical EAE Scoring

2.5.5 Ex-vivo recall assay

Post-mortem, the inguinal lymph nodes (adjacent to the site of immunisation) and spleen of experimental animals were excised and a single cell suspension was

prepared as described below (2.7.1). Following red blood cell lysis, cells were washed twice in cRPMI, counted on a haemocytometer and resuspended at a final concentration of 8 x10^6 cells/ml (spleen) or 6 x10^6 cell/ml (lymph node). For the pMOG EAE system, a ½log dilution series of peptide was established in a 96 well plate with a top concentration of 30 µM and 100 µl of cells was added to each well. A similar arrangement was prepared for the Tg4 system, but with a ½log dilution series commencing with 1 µM Ac1-$9_{(4Y)}$. Cells were incubated at 37 ˚C (5% CO_2) for 72 hours, whereupon supernatants were harvested for cytokine analysis by ELISA.

2.6 T Cell Transfer Model of Colitis

2.6.1 Induction of colitis

Naïve effector $CD4^+$ T cells were isolated from the spleen and peripheral lymph nodes of Foxp3-GFP reporter mice by positive magnet-assisted cell sorting (MACS, Section 2.8.3) selection for $CD4^+$, followed by fluorescence-activated cell sorting (FACS, Section 2.9.4), gating: $CD4^+CD25^-GFP^-$. Following isolation, 5x10^5 T cells were adoptively transferred into $RAG1^{-/-}$ recipients. Animals were monitored regularly throughout the experimental period, at the end of which colonic specimens were prepared for histological assessment.

2.6.2 Pathology scoring

Scoring of histology specimens was performed in a blinded fashion by Dr Mark Arends (consultant histopathologist). Specimens were accorded a combined histology severity score, comprising a score of 0-3 based on each of the following six parameters: crypt architecture, ulceration, crypt abscesses, goblet cell loss, mucosal inflammatory infiltration and submucosal inflammatory infiltration.

2.6.3 Disease Activity Index scoring

Following adoptive transfer of naïve $CD4^+$ T cells, $RAG1^{-/-}$ recipient mice were monitored regularly and accorded a Disease Activity Index score (Table 2-2) to aid objective comparison of the clinical progression of disease.

Colitis Disease Activity Index **(Cumulative score of four parameters, to a maximum of 16)**				
Score	**Weight Loss**	**General Appearance**	**Bleeding**	**Stool Consistency**
0	None (< 1%)	Normal	None	Normal
1	1-5%	Piloerection only	Blood present in faeces	Semi-formed
2	5-10%	Piloerection and lethargy	Blood visible in perianal region	Loose / some blood
3	10-20%	-	-	Diarrhoea adherent to the anus
4	>20%	Motionless or ataxia	Blood visible on fur	Diarrhoea not adherent to the anus

Table 2-2 Colitis Disease Activity Index

2.7 Continuous Infusion via Osmotic Minipump

Alzet® minipumps (supplied by Charles River UK) of 100 µl capacity were selected according to the duration of infusion required for individual experiments (model 1007D – 7 days; model – 1002 – 14 days; model 1004 – 28 days). Minipumps were filled with the substance for infusion (HES, TGM or PBS control) and primed overnight by incubation in PBS at 37°C.

Under general anaesthesia (as per section 2.4.1), abdominal fur was removed by shaving and the skin was prepared with chlorahexidine solution (Animal Care Ltd). The peritoneal cavity was accessed through an upper midline incision and the minipump was placed in the right paracolic gutter. Closure was in two layers with 5-0 undyed Vicryl® (Ethicon UK).

2.8 Cell Isolation and Culture

2.8.1 Preparation of single cell suspensions

Freshly isolated spleen and lymph node specimens were macerated through 70 μm filters (BD) into solution with cRPMI. Contaminating red blood cells were removed by resuspending the cells from one spleen in 2 ml of red blood cell lysis buffer and incubating at RT for 2 minutes. Cells were then washed with cRPMI and counted on a haemocytometer by trypan blue exclusion.

2.8.2 Isolation of Human Lymphocytes

Fresh peripheral blood was obtained by venepuncture of healthy volunteers under a protocol approved by the University of Edinburgh research ethics committee. Blood was collected into heparinised tubes (BD) and immediately diluted 1:1 with PBS. Ficoll-Paque (GE healthcare) was divided into 15 ml aliquots in separate 50 ml conical Falcon tubes (BD). 20 ml of diluted blood was then slowly added to each tube (aiming for two layers with minimal mixing of the two liquids). The tubes were then centrifuged at 400 × *g* for 40 minutes at RT with no brake.

Following centrifugation, PBMCs were carefully aspirated with a Pasteur pipette at the plasma / ficoll interface and placed into a fresh 50 ml conical Falcon tube. The cells were washed three times by adding 40 ml of cRPMI and then centrifuging at

200 g for 10 minutes (RT). Finally, cells were counted on a haemocytometer in preparation for culture.

2.8.3 $CD4^+$ T Cell Enrichment by Magnetic Sorting

Following preparation and counting of cells as described above, cells were resuspended in MACS buffer at a volume of 45 µl per 10^7 cells. 5 µl of microbeads (T3T4, Miltenyi Biotech) were then added per 10^7 cells and incubated at 4°C for 20 minutes. At the end of the incubation period, cells were washed by centrifuging at 200 g for 5 minutes and then resuspending in 10 ml of MACS buffer. Following two washes, cells were resuspended in MACS buffer at a volume of 500 µl per 10^8 cells. $CD4^+$ cells were then isolated by performing a positive selection using an AutoMACS (Miltenyi Biotech) automated magnetic column according to the manufacturer's instructions. The positive fraction of cells was then resuspended in MACS buffer and counted.

2.9 Flow Cytometric Analysis and Cell Sorting

2.9.1 Cell viability and surface staining

Between $4x10^5$ and $2x10^6$ cells were transferred into 5ml round-bottom polystyrene tubes (BD Falcon 352054) and washed twice with 1 ml of PBS (centrifuging samples 200 g for 5 minutes between each wash).

For viability staining, LIVE/DEAD® fixable blue (Life Technologies) was defrosted thoroughly and resuspended PBS at a dilution of 1:1000. A volume of 200 µl of viability staining solution was added to each sample, incubated for 20 min at 4°C (protected from light) and then washed twice in 1 ml of FACS buffer.

To prevent non-specific antigen binding, cells were incubated with 50 µl of polyclonal IgG (diluted 1:50 in FACS buffer) for 10 minutes at 4°C and then washed twice in 1 ml of FACS buffer.

FACS antibodies were diluted to an appropriate final concentration in FACS buffer (table 2-2), to a total volume of 50 µl diluted antibody per $5x10^6$ cells. Single stain controls were individually added to one drop of UltraComp eBeads (eBioscience #01-2222-42). Samples were incubated for 20 min at 4°C, washed twice in 1 ml of FACS buffer and then resuspended in 200 µl FACS buffer for acquisition (if only surface staining is required).

2.9.2 Transcription factor staining

For analysis of transcription factors, cells were resuspended in 400 µl fixation/permeabilisation buffer (eBioscience) and incubated at 4°C for between 1 and 18 hours. Following incubation, cells were resuspended and washed twice in 1 ml permeabilisation buffer (eBioscience). 50 µl of antibody or isotype control (diluted in permeabilisation buffer) was added to each sample. Cells were resuspended by gentle vortex and incubated at room temperature for 30 minutes. Finally, cells were washed in 2 ml of FACS buffer and resuspended in 200 µl FACS buffer for acquisition.

2.9.3 Intracellular cytokine staining

For assessment of intracellular cytokines, cells from the freshly-prepared single cell suspension above (2.8.1) were incubated at 37°C for four hours with PMA (50 ng/ml), ionomycin (1 µg/ml) and Brefeldin A (10 µg/ml) in 200 µl cRPMI. At the end of the culture period, cells were stained for surface markers (as per 2.8.1) and then permeabilised by incubation with Cytofix/Cytoperm solution (BD) for 20 minutes at 4°C. Following incubation, cells were washed twice in Perm/Wash buffer (BD) and then incubated for 20 minutes at 4°C with cytokine-specific antibodies

diluted to an appropriate concentration in Perm/Wash buffer. Finally, cells were washed in 2 ml of Perm/Wash buffer and resuspended in 200 µl FACS buffer for acquisition.

Samples are acquired on a BD Biosciences LSR II or LSR Fortessa flow cytometer and analysed using FlowJo software (Tree Star).

2.9.4 Fluorescence-activated Cell Sorting

$CD4^+$ cells (freshly isolated or from culture) were enriched by magnetic sorting (2.7.3) and then incubated with antibodies for surface markers as described above (2.8.1), but with the omission of a viability stain. Following staining, cells were resuspended in MACS buffer at a concentration of $5x10^8$ cells per ml. Sorting was performed on a BD FACSAria with a gating strategy of: lymphocytes (size exclusion by forward and side scatter), single cells and then stained populations, e.g. $CD4^+CD25^-Foxp3^-CD62L^{hi}$. Cells were sorted into 2 ml of FCS (Gibco) and a sample from each tube was re-acquired on the FACSAria to assess the purity of each sort.

Flow Cytometry Antibodies			
Antigen	**Fluorochrome**	**Clone**	**Manufacturer**
CD3	FITC	17A2	Biolegend
CD4	AF700	RM4-5	Biolegend
CD4	BV650	RM4-5	Biolegend
CD8	PerCP	53-6.7	Biolegend
CD103	Biotin – Streptavidin PerCP	M290	BD Pharmingen
CTLA-4 (CD152)	PE	UC10-4F10-11	BD
PD-1 (CD379)	PE	RMPI-30	eBioscience
CD25	APC	PC61-5	eBioscience
TCR-ß	AF700	H57-597	Biolegend
CD39	PE-Cy7	Duha59	Biolegend
CD44	PE-Cy7	IM7	Biolegend
Foxp3	ef450	FJK-16s	eBioscience
CD45.2	ef450	104	Biolegend

Table 2-3 Flow Cytometry Antibodies

2.10 Proliferation Assays

Cellular proliferation was assessed by generational tracing with CellTrace violet (Life Technologies) or by measurement of thymidine incorporation.

For assessment with CellTrace, MACS purified $CD4^+$ or $CD8^+$ responder cells were washed twice and resuspended in PBS at a concentration of 10^6 cells per ml. Cell Trace was then added to a final concentration of 5 μM and the suspension was agitated thoroughly to ensure uniformity of cell labeling. Labeling cells were incubated for 20 minutes at 37°C, washed once in complete RPMI, then resuspended in 5 ml of complete RPMI and incubated for a further 30 minutes at 37°C to allow the CellTrace to fully incorporate. At the end of the incubation period, cells were washed twice with RPMI and resuspended at a concentration of 10^6 cell per ml; 10^5 cells were then added to each well of a 96 well plate together with stimulation from CD3/CD28 Dynabeads® (Life Technologies, variable concentrations) and varied treatment conditions (e.g. HES).

For assessment of proliferation by thymidine incorporation, whole splenocyte populations or MACS purified responder cells were washed twice and resuspended in complete RPMI at a concentration of 5×10^5 cells per ml. 10^4 responder cells were then added to each well of a 96 well plate. APCs (the unlabelled cells from a $CD4^+$ MACS sort) were irradiated (30 Gy), then immediately washed twice in RPMI and added to culture in the 96 well plate at 1×10^5 cells per well. Stimulation was added to designated wells with soluble CD3 (eBioscience) at a concentration of 2 μg/ml. Finally, varied treatment conditions were added to designated wells. Cells were cultured for 4 days at 37°C and 0.3 μCi [^{3}H] thymidine was added for the final 16 hours of culture (Amersham Biosciences) and measured by β-scintillation counting (Wallac).

2.11 TGF-β Bioassay

A TGF-β bioassay (MFB-F11) was developed by Tesseur *et al* by stably transfecting embryonic fibroblasts from Tgfb1$^{-/-}$ mice with a TGF-β-responsive reporter plasmid containing a secreted alkaline phosphatase reporter gene (SBE-SEAP)[351]. Thus, TGF-βR signaling is proportional to released alkaline phosphatase, which can be measured using an appropriate reagent (e.g. p-nitrophenyl phosphate).

MFB-F11 cells were grown from frozen stocks in 30 ml DMEM with 10 % FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2mM L-glutamine and supplemented with 15 µg/ml Hygromycin B (Invitrogen), for 3 days. Confluent cells were detached from the flask with trypsin, and resuspended in DMEM with 2.5 % FCS, 100 U/ml penicillin, 100 µg/ml streptomycin and 2mM L-glutamine at a concentration of $4x10^5$ cells/ml. In 100 µl, $4x10^4$ cells were added to each well of a 96-well round-bottomed plate. Serial dilutions of test substances (HES, TGM, recombinant human TGF-β1) were then added to each well in a volume of 50 µl and incubated for 24 hours at 37°C. Subsequently, 20 µl of supernatant was aspirated from each well, added to an ELISA plate (NUNC) with 50 µl of reconstituted Sigma FastTM p-nitrophenyl phosphate substrate and incubated at RT in the dark for up to 4 hours. Plates were read on at 405 nm on an Emax precision microplate reader (Molecular Devices).

2.12 Foxp3$^+$ Treg Induction Assay

A single cell suspension was prepared from the spleen and peripheral lymph nodes of Foxp3-GFP transgenic mice. CD4$^+$CD25$^-$GFP$^-$CD62L^{hi} cells were then isolated by MACS followed by FACS sorting (sections 2.7.3 and 2.8.4). Sorted cells were washed twice in complete RPMI and then resuspended in complete RPMI at a concentration of 5 x 10^5 cells per ml. CD3/CD28-coated 24 well plates (Costar) were prepared by adding 250 µl per well of CD3 and CD28 (eBioscience), both at 2 µg/ml in PBS, incubating for 2 hours at 37°C and then washing 3 times in PBS. 5 x 10^5 cells were then added to each well in 1 ml of complete RPMI. Each well was made up to final volume of 2 ml complete RPMI, containing variable concentrations of

treatment conditions (eg. TGF-β) and IL-2 (produced in-house) at a final concentration of 100 U/ml. Cells were removed after 96 hours for flow cytometric analysis.

2.13 Treg Suppression Assays

Cells from the Treg induction assay described above were washed in MACS buffer and the $CD4^+CD25^+GFP^+$ Treg population was isolated by FACS sorting. Responder cells ($CD4^+CD25^-GFP^-CD62L^{hi}$) and nTreg ($CD4^+CD25^+GFP^+$) were also isolated from a fresh Foxp3-GFP transgenic mouse single cell suspension (as in section 2.11). 10^4 responder cells were added to each well of a 96 well round-bottomed plate together with 10^5 irradiated APCs, 2 μg/ml soluble CD3 stimulation and a variable concentration of Treg. Proliferation was assessed after 72 hours by thymidine incorporation (as described in section 2.9).

2.14 Macromolecular Crowding Assays

Assays performed by Dr Hannah Woodcock (Chambers Laboratory, UCL) according to a protocol described by Chen *et al*[352]. WI-38 human lung fibroblasts were cultured under crowded conditions for 48 hours at 37°C in DMEM supplemented by 0.4% FCS and variable concentrations of TGM or TGF-β. At the end of the culture period, cells were fixed with methanol and stained for α-smooth muscle actin (AF594) or type I collagen (AF488) and counterstained with DAPI. Image analysis was performed with MetaMorph® Imaging System software (Molecular Devices).

2.15 Histology and Scoring

Skin graft specimens were fixed in 10% buffered formalin solution overnight and then stored in 100% ethanol. Specimens were embedded in paraffin and then cut in 4

μm transverse sections. Haematoxylin and eosin (H&E) staining was then performed under automated protocol with a Gemini varistainer (Thermo Scientific), according to the manufacturer's instructions.

Histological scoring of allograft rejection was performed in a blinded fashion by Dr Thomas Brenn (consultant histopathologist) according to the Zdichavsky score[353] (Figure 3-2, A). Images were captured using a Leica DFC290 compound microscope and Leica Application Suite software.

2.16 Immunohistochemistry

Skin graft specimens were fixed in methacarn (60% methanol, 30% chloroform, 10% glacial acetic acid) overnight and then stored in 100% ethanol. Samples were embedded in paraffin and then cut in 4 μm transverse sections. Paraffin was then removed from sections by immersing slides in Histoclear (Brunel Microscopes Ltd) for 5 mins, and then hydrating through 100%, 95% and 70% ethanol successively.

Antigen retrieval was achieved by incubating slides in citrate buffer (20 mM citric acid + 0.05% Tween 20 at pH6) at 95°C for 20 minutes. Following 2 washes (5 minutes each) in PBS, slides were loaded into Sequenza racks (Fisher Scientific) and incubated with avidin blocking agent (Vector Laboratories) for 10 minutes at RT, followed by 2 wash steps in PBS (5 minutes each). Slides were incubated with biotin blocking agent (Vector Laboratories) for 10 minutes at RT, followed by 2 wash steps in PBS (5 minutes each). Next, slides were incubated for 10 minutes with serum-free protein blocking agent (DAKO) for 10 minutes at RT, followed by 2 wash steps in PBS (5 minutes each). Primary antibodies (anti-Foxp3 clone FJK-16s, eBioscience – 1:50 dilution and anti-CD3 cloneFJK-16s, eBioscience – 1:100 dilution) were then added in a total volume of 125 μl (with DAKO Real antibody diluent) for each slide and incubated overnight at 4°C.

Following 2 wash steps in PBS, secondary antibodies (goat anti-rabbit AF488, Invitrogen – 1:200 dilution and biotinylated goat anti-rat – 1:100 dilution) were

added in a total volume of 125 µl (with DAKO Real antibody diluent) for each slide and incubated for 1 hour at RT followed by 2 wash steps in PBS (5 minutes each). Slides were then incubated with 125 µl of Streptavidin AF594 (1:200 dilution in DAKO Real antibody diluent) overnight at 4°C and then washed twice in PBS before mounting with Permafluor aqueous mounting medium (Thermo Scientific). Slides were left to dry for 1 hour at RT, then stored at 4°C until image capture on a Leica SP5 C microscope.

2.17 *Ex-vivo* Allogeneic Restimulation Assays

For assessment of antigen-specific cytokine profiles, splenocytes were harvested from C57BL/6 mice 21 days after transplantation of a full-thickness BALB/c skin graft. Isolated splenocytes (10^6) were restimulated *ex-vivo* for 72 hours at 37°C in duplicate under multiple conditions including: soluble anti-CD3e stimulating antibody (eBioscience, 1 µg/ml), 10^6 irradiated BALB/c (donor strain alloantigen) splenocytes, 10^6 irradiated CBA (third party strain alloantigen) splenocytes, 10^6 irradiated C57BL/6 (recipient strain syngeneic antigen) splenocytes, or complete RPMI media.

2.18 Detection of Cytokines by ELISA

For measurement of cytokine concentrations by enzyme-linked immunosorbent assay (ELISA), high-affinity 96-well plates (Immunoplate MaxiSorp, NUNC) were first coated by adding appropriate concentrations of monoclonal capture antibodies in 50 µl of carbonate buffer to each well and incubating overnight at 4^oC. The incubating capture antibody was discarded after 16 hours and the plates were then blocked for 2 hours at 37^oC with 200 µl/well of TBS containing 0.05% Tween and 10% FCS.

Plates were washed 5x in TBS with 0.05% Tween, then 40 µl of sample or standard was added to each well and incubated at 4°C overnight. In most instances,

supernatants were then transferred to a second set of plates to allow quantification of other cytokines.

Following incubation, plates were washed five times in TBS containing 0.05% Tween. Diluted biotinylated detection antibodies were added in a volume of 50 µl per well and incubated at 37^{o}C for 1 hour. Plates were then washed again 5x in TBS with 0.05% Tween and 50 µl of streptavidin-alkaline phosphatase (Sigma) was added to each well, followed by incubation at 37^{o}C for a further 45 minutes.

Finally, plates were washed three times with TBS with 0.05% Tween, and twice with distilled water, before adding 100 µl of p-nitrophenyl phosphate (pNPP) substrate to each well. Optical densities were measured using an Emax precision microplate reader (Molecular Devices).

Cytokine ELISA Antibodies					
Cytokine	**Top Standard Concentration**	**Capture Antibody Clone**	**Capture Antibody Concentration**	**Detection Antibody Clone**	**Detection Antibody Concentration**
IFN-γ	50 ng/ml	XMG1-2	0.5 µg/ml	R4-6A2	0.2 µg/ml
IL-4	8 ng/ml	11B11	0.5 µg/ml	BVD6-24G2	0.06 µg/ml
IL-5	25 ng/ml	TRF-K5	0.125 µg/ml	TRF-K4	0.5 µg/ml
IL-10	10 ng/ml	JES5-16E3	0.5 µg/ml	JES5-2A5	0.5 µg/ml
IL-13	10 ng/ml	eBio- 13A	4 µg/ml	eBio-1316H	0.5 µg/ml
IL-17	20 ng/ml	eBio-17CK15A5	0.5 µg/ml	JES5-2A5	0.2 µg/ml

Table 2-4 Cytokine ELISA Antibodies (all supplied by eBioscience)

2.19 Detection of Cytokines by Cytokine Bead Array

For all CBA dilutions and wash steps, filtered FACS buffer was used (0.5% BSA, 0.05% sodium azide in PBS, passed through 0.22 µm syringe filter).

Standards were defrosted from -80°C and prepared as a mixture according to all of the cytokines that were to be measured. Each standard was diluted such that the top standard is at a concentration of 2,500 pg/ml in 200 µl of filtered FACS buffer. Doubling dilutions were then performed for 11 standards.

Serum or culture supernatant samples were then added to a 96 well round-bottomed plate (50 µl of sample per well). CBA beads (BD Biosciences) were vortexed thoroughly before adding 0.2 µl of each bead per well, in a volume of 50 µl filtered FACS buffer (1/250 dilution of each bead population in FACS buffer). The plate was then shaken at 300 rpm for 1 minute, followed by incubation at room temperature for 1 hour.

Detection antibodies for each cytokine were then added to each of the samples and standards at the same concentration as the CBA beads (0.2 µl of each antibody per well, in 50 µl filtered FACS buffer). The plate was then shaken again at 300 rpm for 1 minute and followed by incubation at room temperature for 1 hour.

Finally, all samples and standards were washed twice with 200 µl of filtered FACS buffer and resuspended in 100 µl per well of filtered FACS buffer for aquisition. Samples were acquired on a BD FACSarray or BD Canto flow cytometer and analysed using FlowJo software (Tree Star).

2.20 Protein Analysis by Western Blotting

Following culture under various conditions, cells were resuspended in 1x cell lysis buffer (Cell Signaling Technology), incubated on ice for 15 minutes and then centrifuged at 200 g for 5 minutes at 4°C. Three parts of the supernatant was then added to one part 4x LDS Sample Buffer (Invitrogen) in the presence of 20 mM β-mercaptoethanol and boiled for 10 minutes. Reduced samples and the SeeBlue Plus 2 marker (Invitrogen) were then added to a NuPage 4-12% Bis-Tris pre-cast gel (Life Technologies) and run in 1x MOPS buffer (Invitrogen) at 140 V for 90 minutes. The gel was then transferred to a nitrocellulose membrane (Bio-Rad) in a semi-dry transfer system using transfer buffer (Invitrogen) supplemented with 10% methanol. The membrane was blocked in 10% (w/v) dried skimmed milk in TBS at 4°C for 2 hours with continuous agitation. Following 4 washes (15 minutes each) in TBST, membranes were incubated with primary antibody (Smad2 or phospho-Smad2, Cell Signaling Technology) at a dilution of 1/1000 overnight at 4°C with continuous agitation. Following four further washes with TBST, membranes were incubated with secondary antibody (anti-rabbit IgG-HRP, Cayman Chemicals), diluted in TBST at a concentration of 1:1000, for 1 hour at room temperature. After 4 final washes in TBST, membranes were developed using a FluorChem SP gel imager (Alpha Innotech) and images were acquired on a FluorChem SP gel imager (Alpha Innotech).

2.21 Statistical Analysis

All statistical analyses were performed using Prism 6.0 (Graphpad Software Inc.). For comparisons of two groups of parametric data, a two-tailed, unpaired t test was used. When three or more groups of parametric data were analysed, a one-way ANOVA was used with Dunnett's multiple comparison test. Graft survival was assessed by Kaplan-Meier analysis and statistical significance of difference in survival between experimental groups was determined by a log rank (Mantel-Cox) test. Comparison of groups of non-parametric data was with the Mann-Whitney U test or Kruskal-Wallis test unless otherwise stated. Generally, p values of <0.05 were considered to be significant; the following symbols were used to indicate significance levels: * denoting $p<0.05$, ** denoting $p<0.01$, *** denoting $p<0.001$ and **** denoting $p<0.0001$.

3 Transplantation

3.1 Introduction

Investigation of the ability of helminths to protect transplanted allografts from rejection first required selection of an appropriate model helminth species. Although *H. polygyrus* had not been investigated in this context before (in the limited number of published studies available, summarised in table 1-2), theoretically it offered a number of advantages over other candidate species. First, in contrast to many other helminths, *H. polygyrus* reliably establishes chronic infection in wild type C57BL/6 mice[354]. This allows for a durable infection throughout the likely timeframe of allograft rejection and suggests superior capabilities for immunomodulation of the host immune response. Secondly, *H. polygyrus* and HES have been shown to powerfully induce Foxp3$^+$ Treg[233], an effect that could be fundamentally important in abrogating allograft rejection[96]. Lastly, significant progress in characterising the secreted immumodulators within HES has been made by the Maizels laboratory in recent years[288,296] and therefore the opportunity for a reductionist approach to specific immunological mechanisms was available with this helminth over other species. While two earlier studies from Lee and colleagues reported enhanced experimental allograft survival following infection with *Nippostrongylus brasiliensis*[269,270]), more recent developments with *H. polygyrus* rendered it the more favourable species for this investigation (T. Lee, personal communication).

Full-thickness skin grafting across allogeneic mouse strains is a reliable transplant model that has been used extensively in studies of immunological tolerance and rejection[104,355,356]. A primary limitation of this model is dependence on a microvascular blood supply, making grafts prone to ischaemia. Inflammation arising from an evolving alloimmune response can induce thrombosis that rapidly propagates and results in graft infarction and necrosis, such that rejection of the graft can occur in a relatively stochastic manner compared to an allograft with a macrovascular blood supply. However, skin grafts do generate a robust allogeneic

immune response for mechanistic analysis and can be performed in relatively high numbers with standard anaesthetic and surgical equipment. On adopting the skin grafting model, grafts were initially sutured onto the interscapular region of recipients (following the technique demonstrated by Mr Kourosh Saeb-Parsy, University of Cambridge). However, this approach required individual caging of recipient animals (to prevent graft damage from other animals, as no protective dressing could be applied in this position) and resulted in multiple allograft draining lymph node populations (bilateral occipital and cervical) with a significant degree of individual variability. The model was therefore adapted to use flexible collodion to fix skin grafts to the recipient flank (as described in section 2.4.2), followed by a dressing that remained in place for 7 days. This technique allowed for consistent allograft draining to the ipsilateral inguinal lymph node (for subsequent analysis by flow cytometry) and the ability to co-house graft recipients (necessary for later experiments involving more than 30 mice). All of the results described in this chapter are derived from experiments using the latter technique.

Scoring of skin graft survival was conducted according to established protocols and corroborated by additional experiments with blinded histological scoring of haematoxylin and eosin-stained allograft sections performed by an independent consultant histopathologist. Histological assessment was performed at multiple time points and found to be optimal at 7 days after transplantation (sufficient for a robust allogeneic response to be underway, but not so advanced that extensive necrosis inhibits assessment).

Finally, *in vivo* experiments with HES in the Maizels laboratory had hitherto been performed with administration by intraperitoneal injection or intranasal insufflation. Based on previously published studies, the timeframe for allograft rejection was predicted to be up to 3 weeks (for BALB/c to C57BL/6 skin grafts). In approaching experimental treatment with HES, a number of unknowns existed, including which component immunomodulators were relevant and their relative proportion, duration of action and bioavailability. Delivery by continuous infusion via an implantable osmotic minipump was therefore adopted, with a rate of infusion equivalent to that of 130 adult *H. polygyrus* worms.

This chapter begins with a functional analysis of the impact of treatment with *H. polygyrus* and HES on allograft survival, followed by investigation of the mechanisms behind their alteration of the alloimmune response and finishing with identification of key differences between the two treatments.

3.2 Results

3.2.1 *H. polygyrus* and HES prolong survival of fully allogeneic skin grafts

Full-thickness allogeneic skin grafts were performed from BALB/c tail skin onto the left flank of C57BL/6 recipient mice, as described in section 2.4.2. With simultaneous anaesthesia and preparation of three recipient mice for each donor, skin graft ischaemic time prior to transplantation was maintained at less than 5 minutes for each procedure. Dressings were removed 7 days after transplantation and grafts were then monitored on a daily basis until complete rejection, which was defined as more than 90% necrosis by surface area, or complete dehiscence (Figure 3-1, A).

Mice that were infected with 200 stage 3 *H. polygyrus* larvae immediately prior to skin grafting (n = 28) showed significantly prolonged allograft survival with a median survival time of 14 days compared to 10 days for mice with identical allografts and no additional treatment (n = 26), $p < 0.0001$ as assessed by the Mantel-Cox (log-rank) test. The hazard ratio (the risk of future rejection in a treatment group expressed as a ratio of the risk of rejection occurring in an untreated group) for *H. polygyrus* infection was 0.365 (95% CI: 0.103-0.368). Control syngeneic (C57BL/6 to C57BL/6) skin grafts showed no signs at all of rejection at the termination of experiments on day 21 post transplantation (n = 6), thereby confirming that destruction of the allografts is as a result of the alloimmune response (Figure 3-1, B).

It is possible that allograft protection afforded by *H. polygyrus* infection may be as a result of the physical presence of the parasite and the response to the intestinal trauma that it elicits (e.g. induction of a Th2 response and alteration of the gut microbiome). To assess this, further experiments were performed with an additional group of mice to which HES was administered via an osmotic minipump (Alzet® model 1004; n = 13) that was implanted into the peritoneal cavity immediately prior to skin grafting. The minipumps eluted 2.64 μg of HES per day for the duration of the experiment (0.11 μl per hour of HES at a concentration of 1 mg/ml). Treatment

with HES significantly prolonged allograft survival in a similar fashion to infection with live *H. polygyrus* larvae, with a median survival time of 14 days compared to 10 days for untreated allograft recipients ($p < 0.0001$ as assessed by the Mantel-Cox test) and a hazard ratio of 0.323 (95% CI: 0.05 – 0.301) (Figure 3-1, C).

3.2.2 *H. polygyrus* and HES ameliorate histological features of fully-allogeneic skin graft rejection

To verify the observation of enhanced allograft survival following treatment with *H. polygyrus* infection or HES shown in Figure 3-1, further experiments were performed to allow for histological assessment of allografts 7 days after transplantation. Scoring was performed in a blinded fashion by Dr Thomas Brenn (consultant histopathologist) according to the Zdichavsky histology scale, which is based on the degree of vasculitis, folliculitis, dermal inflammation, and epidermal degeneration present[353] (Figure 3-2, A-B).

Full-thickness skin grafts were harvested 7 days after transplantation, fixed in 10% formalin for 18 hours and then embedded in paraffin for haematoxylin and eosin staining (Figure 3-2, B). Animals were divided into the following experimental groups (over two independent experiments): BALB/c to C57BL/6 allograft with no treatment (n = 14), BALB/c to C57BL/6 allograft with simultaneous implantation of an intraperitoneal osmotic minipump eluting 2.6 μg/day of HES (Alzet® model 1004; n = 12) and BALB/c to C57BL/6 skin graft with simultaneous infection with 200 *H. polygyrus* stage 3 larvae by oral gavage (n = 10), in addition to C57BL/6 to C57BL/6 syngeneic skin graft controls (n = 5).

Despite the completely normal macroscopic appearance of syngeneic skin grafts up to 21 days after transplantation in the previous experiment, Figure 3-1, B-C), histological analysis at day 7 revealed the presence of sparse areas of inflammation that were sufficiently distinct from normal skin that 4 out of 5 grafts were assigned a grade 1 Zdichavsky score (mean = 0.8 ± 0.2; Figure 3-3, C). Allogeneic grafts with no additional treatment showed at day 7 severe, extensive areas of inflammatory

infiltration and necrosis (despite not having developed >90% macroscopic necrosis until a median of day 10 in the longer-term experiment), resulting in a mean Zdichavsky score of 3.57 ± 0.2; figure 3-3, C. Histological scores of allogeneic grafts onto recipients treated with HES or *H. polygyrus* infection were significantly lower than those that received no treatment (2.25 ± 0.37, p = 0.017 and 1.8 ± 0.36, p = 0.0015, respectively as assessed by the Kruskal-Wallis test).

3.2.3 *H. polygyrus* infection expands intra-allograft regulatory T cell populations

To assess intra-allograft cell populations, BALB/c to C57BL/6 skin grafts were harvested 7 days after transplantation, as before in section 3.2.2, but fixed in methacarn rather than 10% formalin to allow optimal antigen preservation for immunohistochemical staining. Even with methacarn fixation, fluorescent staining of CD4 and CD8 epitopes was not possible. Frozen sections did facilitate CD4 and CD8 staining, but the high proportion of connective tissue in skin led to unacceptably high background staining that did not allow for accurate quantification. A compromise of methacarn-fixed specimens with staining for CD3, Foxp3 and nucleic acids was therefore used (method described in section 2.14). Whilst this allowed for clear identification of co-localisation with low background fluorescence, limitations do exist: $CD3^+Foxp3^+$ cells have been designated as 'Treg' in light of the technical limitations outlined, but could represent either $CD4^+Foxp3^+$ or $CD8^+Foxp3^+$ cells and the relative proportion of $CD4^+$ and $CD8^+$ effector cells is not discernable.

Nonetheless, immunohistochemical analysis of transplanted allogeneic skin grafts revealed a HS change (13.64 fold) in the mean number of $CD3^+Foxp3^+$ cells visible per high-powered field per graft (Figure 3.3, p = 0.0002). Whether these cells are in fact $CD4^+Foxp3^+$ or $CD8^+Foxp3^+$, both have a regulatory phenotype and both are likely to promote allograft survival.

3.2.4 HES expands Foxp3$^+$ Treg populations *in vivo* and PD-1 expression of Foxp3$^-$ effector CD4$^+$ T cells

To investigate the influence of HES on the alloimmune response, allogeneic (BALB/c to C57BL/6) full-thickness skin grafts were performed with recipient animals receiving either no additional treatment, or placement of a subcutaneous osmotic minipump eluting 2.6 μg/day of HES. Flow cytometric analysis of the allograft draining (inguinal) lymph node cellular composition revealed that treatment with HES resulted in a 42% increase in CD4$^+$CD25$^+$Foxp3$^+$ Treg (as a percentage of all CD4$^+$ cells; $p = 0.009$; Figure 3-4, A). Mean fluorescence intensity (MFI) of Foxp3 within the Treg population was also significantly increased with HES treatment (Figure 3-4, B). Analysis of Programmed cell death protein 1 (PD-1) showed increased expression induced by HES that was specific to the Foxp3$^-$ effector CD4$^+$ population (Figure 3-4, C-E). CD4$^+$Foxp3$^-$PD-1$^+$ cells as a proportion of total CD4$^+$ increased by 62% following treatment with HES ($p = 0.03$; Figure 3-4, C), whilst expression of PD-1 within the CD4+Foxp3+ compartment was not significantly changed ($p = 0.2$; Figure 3-4, D).

Following the use of subcutaneous implantation of minipumps in this experiment, the technique was modified to intraperitoneal placement for improved systemic absorption and reduced variability in the localised concentration of HES to which individual subcutaneous lymph node populations were exposed (a particularly important point for later experiments with animals immunised by bilateral hind limb injection).

3.2.5 *H. polygyrus* and HES suppress peripheral blood serum inflammatory cytokines following allogeneic skin grafting

To further investigate immunological effects of HES on the allogeneic response, serum cytokines were analysed. BALB/c full-thickness skin grafts were transplanted on to C57BL/6 recipient mice in the following experimental groups: no treatment (n

= 8), insertion of an intraperitoneal osmotic minipump eluting 2.5 μg/day of HES immediately prior to transplantation (n = 6), or infection with 200 *H. polygyrus* stage 3 larvae immediately prior to transplantation (n = 7). C57BL/6 to C57BL/6 skin grafts were also performed as controls (n = 3).

Seven days after transplantation, peripheral blood was collected from the brachial artery under terminal anaesthesia. Serum from each mouse was then analysed for multiple cytokine concentrations in parallel by cytokine bead array (as described in section 2.16). Treatment with HES significantly suppressed serum concentrations of multiple pro-inflammatory cytokines, notably including characteristic cytokines from Th1, Th2 and Th17 effector $CD4^+$ T cell phenotypes (Figure 3-5). Most notably, serum IL-1α, which showed the strongest upregulation following allograft transplantation, was profoundly suppressed, as were levels of a number of other cytokines such as IL-6, IL-17 and IL-21 that had not been greatly increased in allograft recipients. The mean concentrations of all inflammatory cytokines measured from allografts treated with *H. polygyrus* infection were thus lower than those of untreated allograft recipients. The exception to the trend was with (the Th2 cytokine) IL-5, in which HES did not effect any significant change and *H. polygyrus* infection led to a significantly raised concentration. The concentrations, fold changes and p values (determined by one-way ANOVA with Dunnett's multiple comparison test) are summarised in Table 3-1.

Cytokine	Allograft only Concentration - pg/ml	+HES Concentration - pg/ml (fold change compared to no treatment; p value)	+*H. polygyrus* Concentration - pg/ml (fold change compared to no treatment; p value)
IL-1α	38.17	17.14 (0.5; **p = 0.004**)	27.18 (0.7; p = 0.192)
TNF	33.94	28.68 (0.9; **p = 0.004**)	32.15 (0.9; p = 0.58)
IL-17	18.35	16.36 (0.9; **p = 0.004**)	16.94 (0.9; p = 0.1128)
IL-6	62.32	46.86 (0.8; **p = 0.042**)	59.3 (0.9; p = 0.328)
IL-21	96.64	87.89 (0.9; **p = 0.039**)	90.46 (0.9; p = 0.151)
IFNγ	129.3	113.5 (0.9; p = 0.102)	128.3 (1.0; p = 0.935)
IL-4	37.23	35.54 (0.9; **p = 0.015**)	35.88 (1.0; p = 0.084)
IL-5	30.53	31.51 (1.0; p = 0.528)	37.29 (1.2; **p = 0.014**)

Table 3-1 Serum cytokines 7 days after BALB/c to C57BL/6 full-thickness skin grafts (n=8 per group)

3.2.6 *H. polygyrus*-infected allogeneic skin graft recipients develop a shift from a Th1 and Th17 allogeneic response to Th2 upon restimulation

To evaluate alloantigen-specific cytokine responses, as distinct from the systemic cytokine milieu in peripheral blood serum, splenocytes were harvested from uninfected and *H. polygyrus*-infected allograft recipients 21 days after transplantation and restimulated with irradiated donor allogeneic cells for 72 hours at 37°C (Figure 3-4, A; method described in section 2.17).

Analysis of culture supernatants by ELISA revealed significantly reduced IFNγ and IL-17 production from splenocytes of *H. polygyrus*-infected allograft recipients compared to those from untreated allograft recipients. This effect was most

pronounced at higher concentrations of stimulating allogeneic cells; with 10^6 BALB/c stimulating cells, the mean IFNγ concentration arising from *H. polygyrus*-infected allograft recipients was 2.41-fold lower than that from non-infected allograft recipients ($p = 0.0004$; Figure 3-14, B). The trend for IL-17 was the same, with 10^6 BALB/c stimulating cells inducing a 4.21-fold reduction in IL-17 from *H. polygyrus* infected allograft recipients compared to uninfected controls ($p < 0.0001$; Figure 3-4, C).

The relationship between *H. polygyrus* infection and cytokine production upon allogeneic restimulation was completely reversed with respect to IL-4: 10^6 BALB/c stimulating cells induced a 4.3-fold higher response from *H. polygyrus* infected allograft recipients compared to uninfected controls ($p < 0.0006$; Figure 3-4, D). This suggests that instead of *H. polygyrus* infection merely inducing a parasite-specific Th2 response in parallel to a separate anti-allograft response, concomitant infection actually alters the cytokine profile of the alloantigen-specific response itself.

3.2.7 *H. polygyrus*-induced Th2 shift is donor alloantigen-specific

Further to the finding of a shift towards a Th2 response upon restimulation of splenocytes from *H. polygyrus*-infected allograft recipients with donor alloantigens, additional experiments were performed to compare this response to that against third-party alloantigens.

Full-thickness skin grafting was performed from BALB/c donors onto C57BL/6 recipients as before with either no treatment (n = 5) or simultaneous infection with 200 *H. polygyrus* stage 3 larvae by oral gavage (n = 6). Splenocytes were harvested from recipient animals 21 days after transplantation and restimulated *ex vivo* with syngeneic, donor allogeneic (BALB/c) or third party allogeneic (CBA) cells in parallel for 72 hours at 37°C, alongside anti-CD3 polyclonal stimuli (Figure 3-5, A). The concentrations of IFNγ, IL-4 and IL-10 in culture supernatants were then measured by ELISA.

Restimulation with irradiated BALB/c (donor strain) splenocytes revealed the same cytokine profile as previous experiments (as shown in Figure 3-4): the mean IFNγ concentration arising from splenocytes of *H. polygyrus*-infected allograft recipients was 53% lower than that of untreated allograft recipients ($p = 0.007$; Figure 3-5, B), whilst the mean IL-4 production from *H. polygyrus*-infected allograft recipients was reciprocally increased (2.0-fold change, $p = 0.018$; Figure 3-5, C)

Following restimulation with irradiated CBA (third party allogeneic) cells, responses were generally much lower than with donor cells. However, a measurable IFNγ response was induced in splenocytes from allograft recipients, which was 56% lower in spleen cells from those treated with *H. polygyrus* infection ($p = 0.003$; Figure 3-5, B). In contrast to stimulation with donor strain BALB/c cells, no significant difference in IL-4 production was observed (0.16 ± 0.005 ng/ml vs. 0.17 ± 0.004 ng/ml, $p = 0.91$; figure 3-5, C). Thus, it appears that the 'Th2 switch' previously observed upon allogeneic restimulation of splenocytes from *H. polygyrus*-infected skin graft recipients is alloantigen-specific for the skin graft donor strain.

Polyclonal stimulation with soluble CD3 revealed a 65.21% reduction in IFNγ production from *H. polygyrus*-infected allograft recipient cells compared to untreated allograft recipients ($p = 0.0001$; Figure 3-5, B) and a corresponding 4.1-fold increase in IL-4 production was also seen ($p = 0.012$; Figure 3-5, C).

In respect of IL-10, only restimulation with CD3 revealed a significant difference between *H. polygyrus*-infected allograft recipient cells compared to untreated allograft recipients, with a substantial increase observed (0.63 ± 0.13 ng/ml vs. 0.16 ± 0.04 ng/ml, $p = 0.009$; Figure 3-5, D).

Control conditions of culture with 10^6 irradiated syngeneic C57BL/6 splenocytes or an equal volume of complete RPMI media revealed no significant difference in production of IFNγ, IL-4 or IL-10 between experimental groups (Figure 3-5, B-D).

3.2.8 HES-mediated allograft protection is not Th2-dependent

Induction of a Th2 cytokine profile in alloreactive lymphocytes has previously been suggested as a possible mechanism behind *Nippostrongylus brasiliensis*-mediated protection of allogeneic mouse cardiac allografts[269]. Following the finding that allogeneic skin grafting with concomitant *H. polygyrus* infection induces a Th2 response that is specific to donor alloantigens (section 3.2.7), a further experiment was performed with parallel wild type and IL-4 receptor deficient (IL-4Rα$^{-/-}$) donor and recipient mice.

Full-thickness wild type BALB/c skin grafts were transplanted on to wild type C57BL/6 recipients with no treatment (n = 6), insertion of an intraperitoneal osmotic minipump eluting 2.5 μg/day of HES (n = 7), or infection with 200 *H. polygyrus* stage 3 larvae (n = 8). In parallel, skin grafts from BALB/c IL-4Rα$^{-/-}$ donors were transplanted onto C57BL/6 IL-4Rα$^{-/-}$ recipients with either no treatment (n = 5) or insertion of an intraperitoneal osmotic minipump eluting 2.5 μg/day of HES (n = 4). An experimental group of IL-4Rα$^{-/-}$ mice receiving infection with *H. polygyrus* was not included due to a severely limited availability of these transgenic strains; treatment with HES was chosen over *H. polygyrus* infection because of concern that the greatly increased susceptibility of IL-4R-deficient mice to infection might make comparison of effects with the wild-type more difficult to interpret

Kaplan-Meier analysis of the time for transplanted skin grafts to completely reject (>90% necrosis by surface area or complete dehiscence) revealed a 40% increase in the median survival of allografts onto wild-type recipients treated with HES (hazard ratio = 0.247, p = 0.0003) and *H. polygyrus* infection (hazard ratio = 0.304; p = 0.002) (Figure 3-8, A). Similarly, median survival of BALB/c IL-4Rα$^{-/-}$ skin grafts onto C57BL/6 IL-4Rα$^{-/-}$ recipients was 44.45% longer with HES treatment compared to untreated controls (hazard ratio 0.347; p = 0.046) (Figure 3-8, B). Inherent variability within the skin grafting model (e.g. trauma caused by individual mice attempting to remove dressings, etc) generally necessitates greater experimental numbers than were available for this experiment for robust conclusions to be drawn.

However, from the limited data available, it does appear that HES-mediated allograft protection is not wholly dependent on a Th2 alloimmune response.

Flow cytometric analysis of the allograft draining lymph node revealed a significant increase in $Foxp3^+$ Treg with HES treatment compared to untreated allograft recipients, both in wild type animals (1.16 fold change; $p = 0.012$) and IL-$4R\alpha^{-/-}$ (1.26 fold change; $p = 0.019$) (Figure 3-8, C+E). The proportion of Treg was not significantly increased in the spleens of allograft recipients treated with HES compared to untreated recipients, in wild type mice but was in IL-$4R\alpha^{-/-}$ animals (1.2 fold change; $p = 0.03$) (Figure 3-8, D).

3.2.9 *H. polygyrus* infection and HES suppress expression of Tbet by $CD4^+$ T cells

As discussed in section 1.1.3.4, Th1 $CD4^+$ T cells play a fundamental role in mediating allograft rejection. To assess the impact of *H. polygyrus* and HES in mitigating against an allogeneic Th1 response, cell populations from the spleen and allograft draining lymph node of skin graft recipient animals were analysed by flow cytometry for expression of the transcription factor Tbet. Two experiments were performed under identical conditions to allow for cellular analysis at 7 and 21 days after transplantation.

Full-thickness BALB/c skin grafts were transplanted on to C57BL/6 mice in the following experimental groups: no treatment, implantation of an intraperitoneal osmotic minipump eluting 2.6 μg/day of HES immediately prior to transplantation and infection with 200 *H. polygyrus* stage 3 larvae by oral gavage immediately prior to transplantation. Syngeneic C57BL/6 to C57BL/6 skin grafts were also performed in each experiment to provide control cell populations.

Tbet expression by $CD4^+$ T cells was significantly elevated in the spleen and allograft draining lymph node of all mice that received an allogeneic graft with no treatment compared to syngeneic control skin grafts (Figure 3-9, A-D). Changes in

Tbet expression were observed as a shift in fluorescence intensity and so positive populations were determined by gating against a fluorescence minus one control (Figure 3-9, E).

HES and *H. polygyrus* infection were both found to suppress Tbet expression by $CD4^+$ T cells in the spleen and allograft draining lymph node. A summary of the mean proportions of $CD4^+Tbet^+$ T cells, together with fold change as a result of treatment and associated p values (determined by one-way ANOVA with Dunnett's multiple comparisons test) is presented in Table 3-2.

Cell population	Allograft only %$CD4^+Tbet^+$ of all $CD4^+$ cells	+HES %$CD4^+Tbet^+$ (fold change compared to no treatment; p value)	+*H. polygyrus* %$CD4^+Tbet^+$ (fold change compared to no treatment; p value)
Day 7 LN	61.75	44.84 (0.73; **p = 0.0012**)	40.23 (0.65; **p < 0.0001**)
Day 7 Spleen	19.72	11.23 (0.57; **p = 0.0001**)	12.29 (0.62; **p = 0.0003**)
Day 21 LN	59.62	44.9 (0.75; **p = 0.0016**)	34.75 (0.58; **p < 0.0001**)
Day 21 Spleen	52.79	44.83 (0.85; p = 0.0844)	37.97 (0.72; **p = 0.0019**)

Table 3-2 Tbet expression by $CD4^+$ T cells of allograft recipients (comparison following treatment with *H. polygyrus* and HES)

3.2.10 *H. polygyrus* infection and HES suppress expression of ROR-γt by $CD4^+$ T cells

Th17 $CD4^+$ T cells have also been shown to mediate allograft rejection in multiple experimental transplant models[82,83,86] (as discussed in section 1.1.3.6). Given that HES has previously been shown to exert TGF-β-like activity[233] and that the Th17 phenotype can be generated *in vitro* from naïve $CD4^+$ T cells stimulated in the presence of TGF-β and IL-6, one might reasonably hypothesise that HES (and *H. polygyrus*) could expand harmful alloreactive Th17 cells within the inflammatory context of a rejecting allograft.

To address this concern, the $CD4^+$ T cell populations isolated from allograft recipient animals described in 3.2.9 (from spleen and allograft draining lymph node, with or without treatment with HES or *H. polygyrus*) were stained for expression of the Th17 transcription factor, ROR-γt (Figure 3-10). Compared to recipients of a syngeneic skin graft, ROR-γt expression was markedly elevated in untreated allograft recipients 21 days after transplantation (2.16 fold change in allograft draining lymph node $CD4^+$ T cells, $p = 0.0002$, and a 2.63 fold change in splenic $CD4^+$ T cells, $p = 0.002$). Treatment with *H. polygyrus* infection suppressed ROR-γt expression in $CD4^+$ T cells within the spleen and lymph node at both day 7 and day 21 post-transplantation. Likewise, treatment with HES suppressed expression of ROR-γt by splenic $CD4^+$ T cells at 21 days post transplantation and did not increase ROR-γt expression in the lymph node or spleen at the other time points assessed. A summary of the mean proportions of $CD4^+$ROR-γt^+ T cells, fold change with treatment and associated p values (determined by one-way ANOVA with Dunnett's multiple comparisons test) is presented in table 3-3.

Cell population	Allograft only %CD4$^+$ROR-γt$^+$ of all CD4$^+$ cells	+HES %CD4$^+$ROR-γt$^+$ (fold change compared to no treatment; p value)	+*H. polygyrus* %CD4$^+$ROR-γt$^+$ (fold change compared to no treatment; p value)
Day 7 LN	6.19	5.1 (0.82; p = 0.1932)	5.0 (0.81; p = **0.0298**)
Day 7 Spleen	6.24	5.4 (0.86; p = 0.1461)	4.9 (0.79; p = **0.0215**)
Day 21 LN	26.85	17.4 (0.65; p = 0.2383)	9.5 (0.35; p = **0.0215**)
Day 21 Spleen	14.93	7.5 (0.5; **p = 0.0032**)	5.6 (0.37; p = **0.0004**)

Table 3-3 ROR-γt expression by CD4$^+$ T cells of allograft recipients (comparison following treatment with *H. polygyrus* and HES)

3.2.11 *H. polygyrus* infection and HES exert different effects on expression of GATA3 by splenic CD4$^+$T cells

In light of the known ability of infection with *H. polygyrus* to induce a strong Th2 response[357] and the novel finding of its ability to polarise the alloimmune response towards a Th2 phenotype, a plausible hypothesis at this stage might be that suppression of Th1 and Th17 alloimmune responses by *H. polygyrus* and HES were as a result cross-regulation from a simultaneous Th2 response. However, both *H. polygyrus* and HES also induce Foxp3$^+$ Treg (Figure 3-8, C) and, crucially, the ability of HES to protect allografts from rejection is preserved in IL-4Rα$^{-/-}$ mice.

CD4$^+$ T cells isolated from allograft recipient mice described in 3.2.9 (receiving no treatment, continuous HES infusion, or infection with *H. polygyrus*) were further analysed for expression of GATA3 to identify the proportion of cells that had differentiated into the Th2 phenotype. Within the allograft draining lymph node, treatment with both HES and *H. polygyrus* infection suppressed GATA3 expression compared to untreated allograft recipients (0.68 fold change, p = 0.0164 and 0.48 fold change, p = 0.0004, respectively) (Figure 3-11, A). However, within the splenic CD4$^+$ T cell population, treatment with HES and *H. polygyrus* precipitated opposite

effects on GATA3 expression: whilst HES was suppressive (0.78 fold change, $p = 0.011$), *H. polygyrus* infection induced a marked increase (1.77 fold change, $p < 0.0001$) (Figure 3-11, B+C).

GATA3 expression by $CD4^+$ T cells was significantly elevated in untreated allograft recipients compared to syngeneic skin graft recipient control mice, both in the allograft draining lymph node (3.16 fold change, $p < 0.0001$) and spleen (1.57 fold change, $p = 0.0006$) (Figure 3-11, A+B).

3.2.12 *H. polygyrus* infection induces a Th2 shift that is donor alloantigen-specific; HES does not

Following the finding of differential effects of HES and *H. polygyrus* infection on GATA3 expression by $CD4^+$ T cells (Figure 3-11, B), splenocytes harvested from allograft recipients 21 days after transplantation were restimulated under multiple conditions in parallel for 72 hours at 37°C. Experimental conditions were the same as those described in section 3.2.9 (allografts with no treatment, continuous HES infusion, or infection with *H. polygyrus*, in addition to syngeneic graft controls).

Restimulation with irradiated BALB/c (donor allogeneic) cells revealed a significant reduction of IFN-γ production (measured in the culture supernatant by ELISA) by splenocytes from allograft recipients treated with HES compared to those that received no treatment (0.58 fold change, $p = 0.0005$). IFN-γ production by splenocytes from *H. polygyrus*-treated allograft recipients compared to untreated allograft recipients was even further reduced (0.47 fold change, $p < 0.0001$). This pattern was similar to that of IFN-γ production following restimulation with soluble CD3 (Figure 3-12, A) and to expression of Tbet by $CD4^+$ T cells 21 days after transplantation (Figure 3-9, B+D).

Measurement of IL-4 following restimulation with irradiated BALB/c (donor allogeneic) cells revealed a marked increase in production by splenocytes from *H. polygyrus*-treated allograft recipients compared to untreated allograft recipients (6.02

fold change, p = 0.0002; Figure 3-12, B). However, no increase in IL-4 production was seen in splenocytes from HES-treated allograft recipients under the same restimulation conditions (0.28 fold change, p = 0.46). This finding provides corroborative evidence of the key difference between treatment with HES and *H. polygyrus* infection in inducing Th2 differentiation indicated by GATA3 expression (Figure 3-11, B) and, further, indicates that this difference in $CD4^+$ phenotype induction affects the alloreactive T cell population.

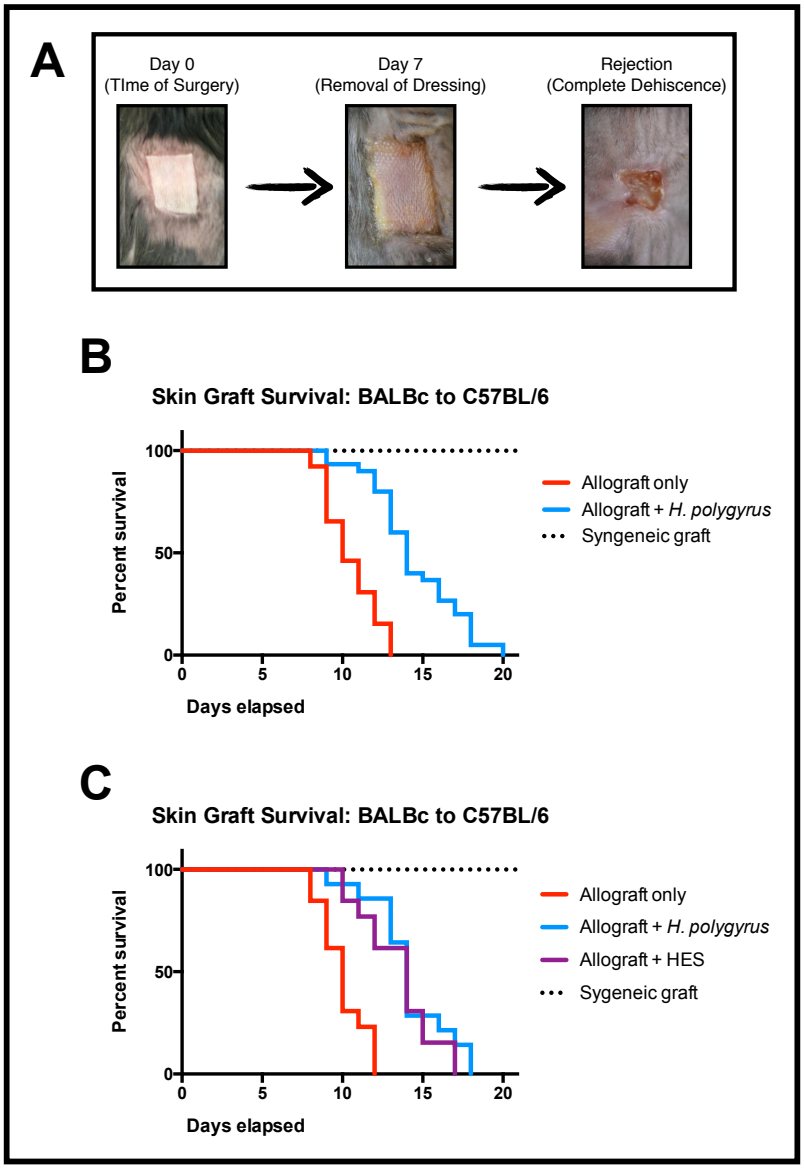


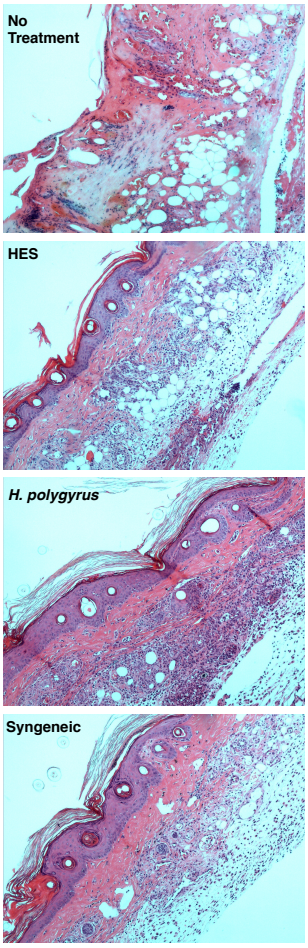
Figure 3-1 *H. polygyrus* and HES prolong survival of fully-allogeneic skin grafts
(A) Appearances of full-thickness BALB/c to C57BL/6 full-thickness skin grafts. Grafts of 1 cm^2 tail skin were transplanted onto the recipient animal's left flank and monitored daily from day 7 (upon removal of dressings) onwards. The end point for rejection was allograft dehiscence (as shown) or complete necrosis of >95% of the allograft surface area.
(B) Kaplan-Meier curve of full-thickness skin graft survival: allograft only (BALB/c to C57BL/6 skin graft, n = 26), allograft + *H. polygryus* (BALB/c to C57BL/6 skin graft with simultaneous infection with 200 *H. polygyrus* stage 3 larvae by oral gavage, n = 28), and syngeneic grafts (C57BL/6 to C57BL/6 skin graft controls, n = 6). Mantel-Cox comparison of allograft vs. allograft + *H. polygyrus* survival curves: $p < 0.0001$. Data shown comprised of four independent experiments.
(C) Kaplan-Meier curve of full-thickness skin graft survival: allograft only (BALB/c to C57BL/6 skin graft, n = 13), allograft + *H. polygryus* (BALB/c to C57BL/6 skin graft with simultaneous infection with 200 *H. polygyrus* stage 3 larvae by oral gavage, n = 14), allograft + HES (BALB/c to C57BL/6 skin graft with simultaneous implantation of an intraperitoneal osmotic minipump eluting 2.6 μg/day of HES, n = 13) and syngeneic grafts (C57BL/6 to C57BL/6 skin graft controls, n = 3). Mantel-Cox comparison of allograft vs. allograft + HES survival curves: $p = 0.0002$. Data shown is comprised of two independent experiments.

A

Grade	Vasculitis	Folliculitis	Dermal inflammation	Epidermal degeneration
0	None	None	None	None
1	None/mild	None/mild	Mild	None/mild
2	Moderate focal	Mild/moderate	Mild/moderate	Mild
3	Moderate diffuse	Moderate	Moderate	Moderate
4	Severe/necrosis	Severe/necrosis	Severe/necrosis	Severe/necrosis

Zdichavsky *et al.* Scoring of Skin Rejection in a Swine Composite Tissue Allograft Model, J Surg Res, 1998.85(1)1-8

B



C

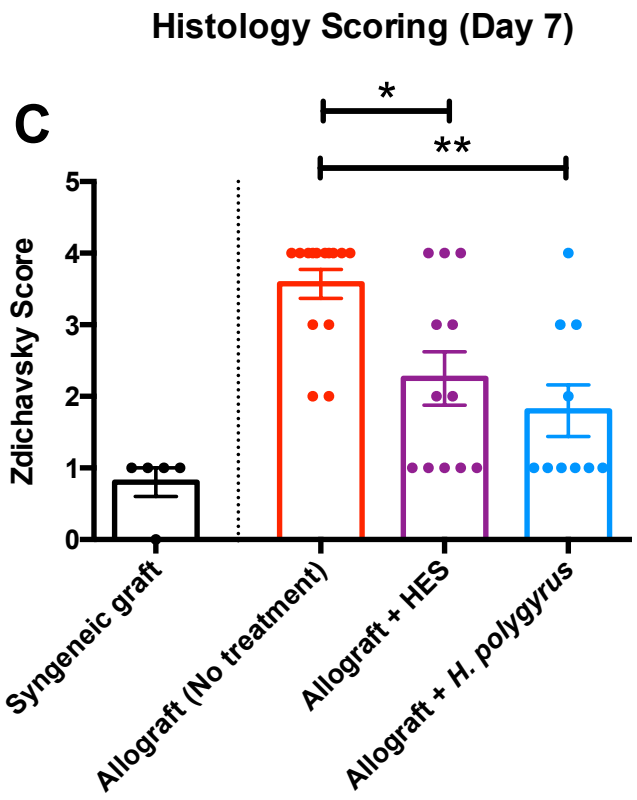


Figure 3-2 *H. polygyrus* and HES ameliorate histological features of fully-allogeneic skin graft rejection

(A) Zdichavsky histological score of allograft rejection (each graft is accorded a score according to the mean value for the four parameters shown).

(B) Representative haematoxylin and eosin-stained histology sections of skin grafts 7 days after transplantation (from top: BALB/c to C57BL/6 allograft with no treatment, BALB/c to C57BL/6 allograft with simultaneous implantation of intraperitoneal osmotic minipump eluting 2.6 μg/day of HES, BALB/c to C57BL/6 skin graft with simultaneous infection with 200 *H. polygyrus* stage 3 larvae by oral gavage and C57BL/6 to C57BL/6 syngeneic skin graft controls.

(C) Zdichavsky scores (mean ± SEM) of the experimental groups described in (B); statistical significance assessed by the Kruskal-Wallis test. Data shown is comprised of two independent experiments. Scoring was performed in a blinded fashion by Dr Thomas Brenn, consultant histopathologist.

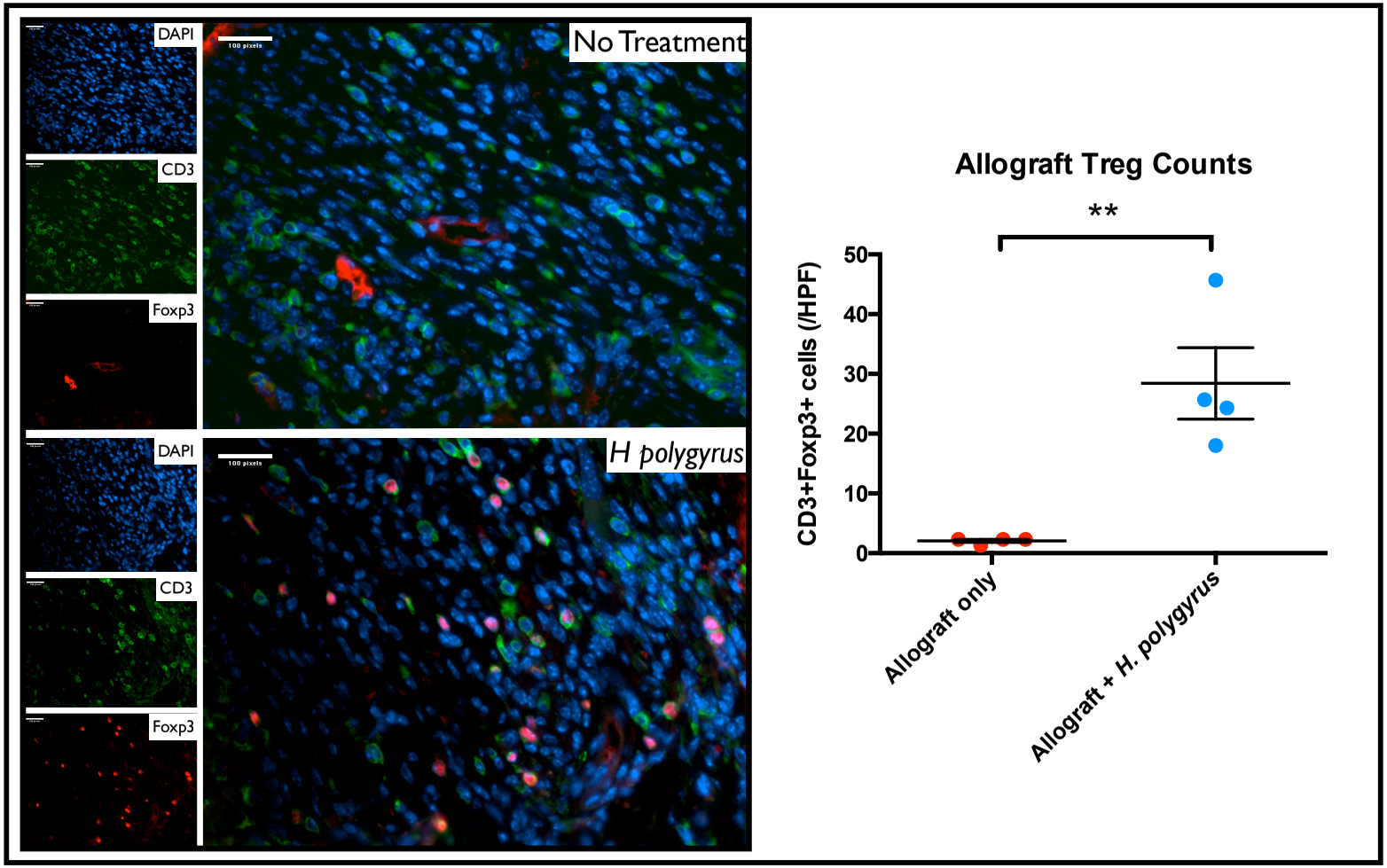


Figure 3-3 *H. polygyrus* infection expands intra-allograft regulatory T cell populations
Full-thickness BALB/c to C57BL/6 skin grafts were harvested 7 days after transplantation from two experimental groups - allograft only (no additional treatment; n = 4) and allograft immediately preceded by infection with 200 *H. polygyrus* stage 3 larvae by oral gavage (n =4). Grafts were fixed in methacarn for 18 hours prior to embedding in paraffin. Sections were then stained for Foxp3 (Alexa Fluor® 594), CD3 (Alexa Fluor® 488) and nucleic acids (DAPI). Positively stained cells were counted in three high-powered fields per graft and a the mean for each graft is plotted above. Statistical significance assessed by unpaired t test (p = 0.0046).

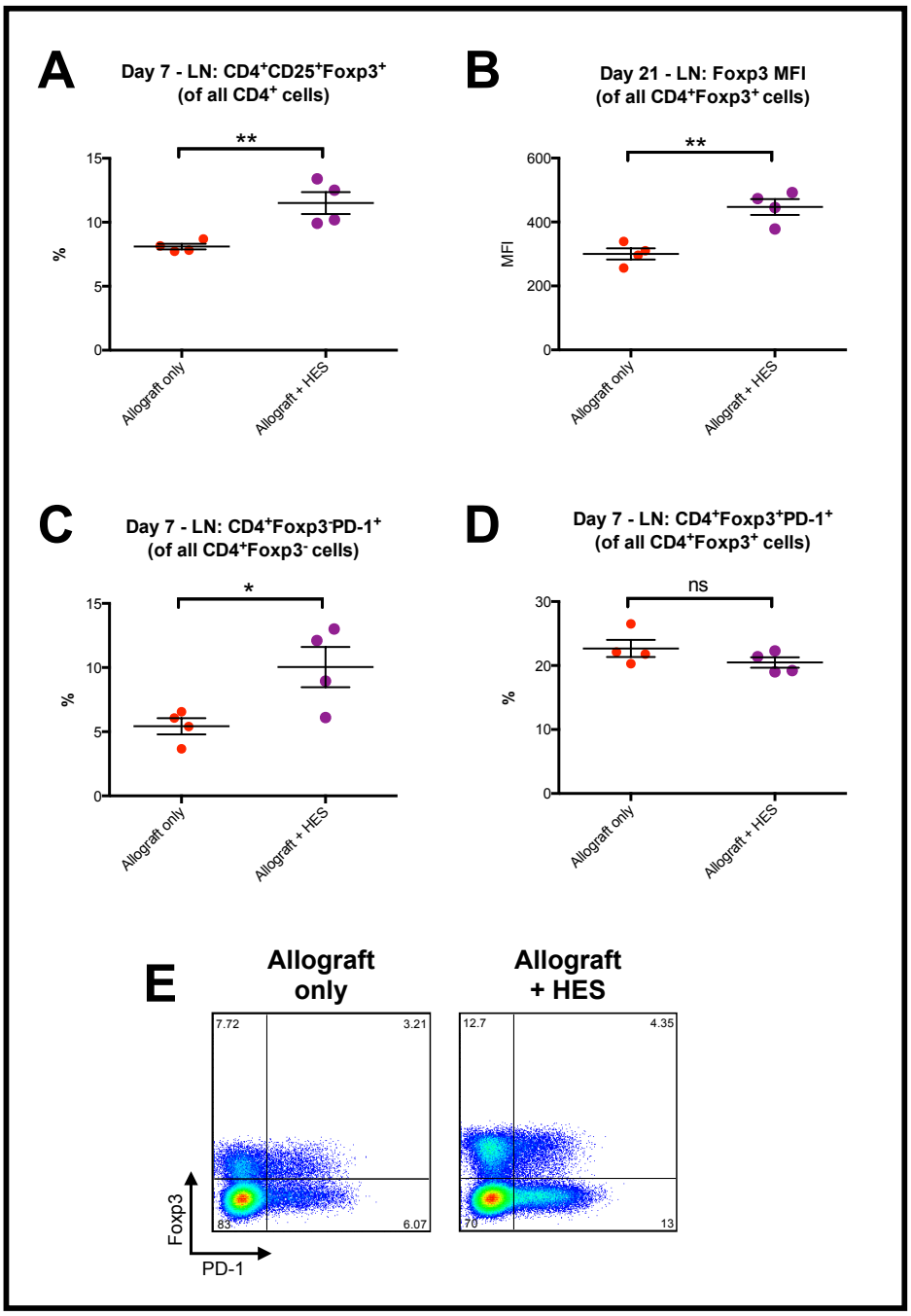


Figure 3-4 HES expands Foxp3$^+$ Treg populations *in vivo* and PD-1 expression by Foxp3$^-$ effector CD4$^+$ T cells

Full-thickness BALB/c to C57BL/6 skin grafts were performed for two experimental groups - allograft only (no additional treatment; n = 4) and allograft immediately preceded by implantation of a subcutaneous osmotic minipump eluting 2.6 μg/day of HES (n =4). Flow cytometric analysis of cells within the allograft draining (inguinal) lymph node are shown. (A) Treg - %CD4$^+$CD25$^+$Foxp3$^+$ cells of all CD4$^+$ cells. (B) Foxp3 mean fluorescence intensity (% of all CD4$^+$Foxp3$^+$ cells). (C) PD-1 expression (% of CD4$^+$Foxp3$^-$ cells). (D) PD-1 expression (% of CD4$^+$Foxp3$^+$ cells). (E) Representative flow cytometry plots from (C).

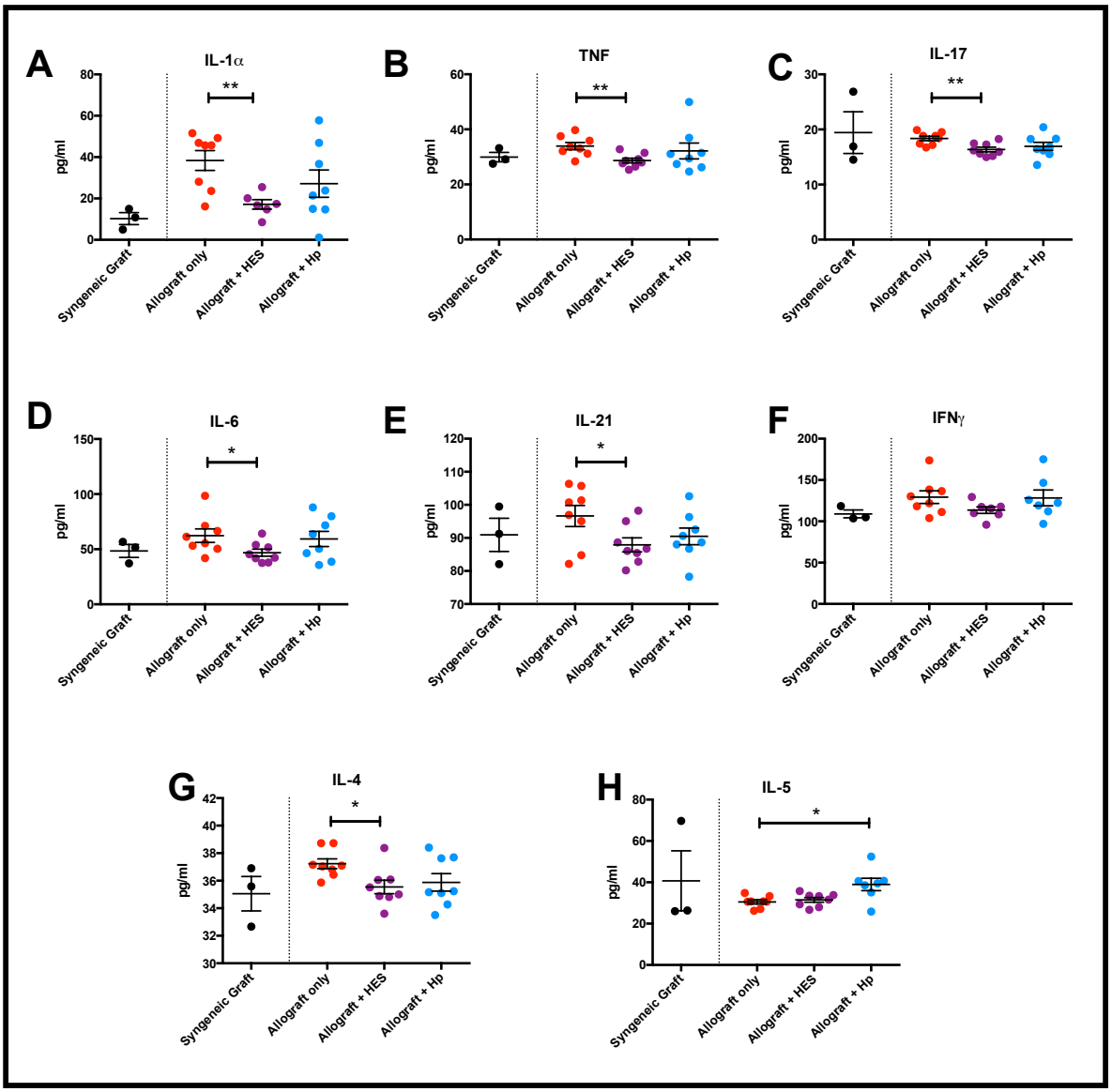


Figure 3-5 *H. polygyrus* and HES suppress peripheral blood serum inflammatory cytokines following allogeneic skin grafting

Full-thickness allogeneic (BALB/c to C57BL/6) and syngeneic control (C57BL/6 to C57BL/6) skin grafts were performed with the following experimental conditions: allograft only, allograft + HES (allograft immediately preceded by implantation of an intraperitoneal osmotic minipump eluting 2.6 μg/day of HES) and allograft + *H. polygyrus* (allograft immediately preceded by infection with 200 *H. polygyrus* stage 3 larvae by oral gavage). Peripheral blood serum cytokines (measured by cytokine bead array) 7 days after transplantation are shown. Statistical significance assessed by one-way ANOVA with Dunnett's multiple comparisons test.

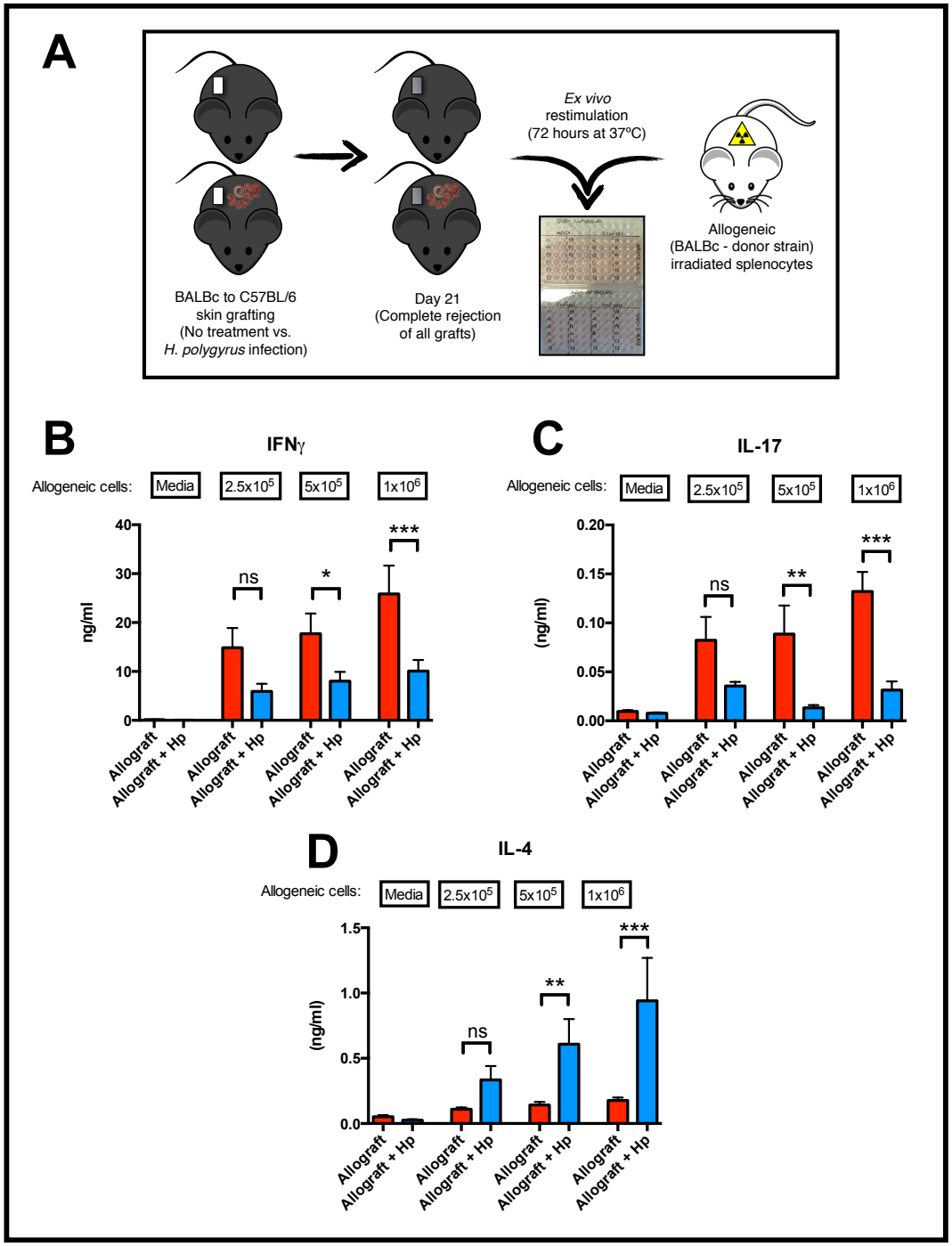


Figure 3-6 *H. polygyrus*-infected allogeneic skin graft recipients develop a shift from a Th1 and Th17 allogeneic response to Th2 upon restimulation

(A) Splenocytes were harvested from C57BL/6 mice 21 days after transplantation of a full-thickness BALB/c skin graft. Allograft recipients either received no treatment (n = 8) or were infected with 200 stage 3 *H. polygyrus* larvae immediately prior to skin grafting (n = 9). Isolated splenocytes (10^6) were restimulated *ex-vivo* for 72 hours at 37°C in duplicate with 2.5 x10^5, 5 x 10^5, 1 x 10^6 irradiated (30 Gy) BALB/c (donor strain alloantigen) splenocytes or complete RPMI media as a control. (B) Restimulation supernatant IFN-γ concentration measured by ELISA. (C) Restimulation supernatant IL-17 concentration measured by ELISA. (D) Restimulation supernatant IL-4 concentration measured by ELISA. Data shown are representative of two similar experiments. Statistical significance assessed by one-way ANOVA with Sidak's multiple comparison test.

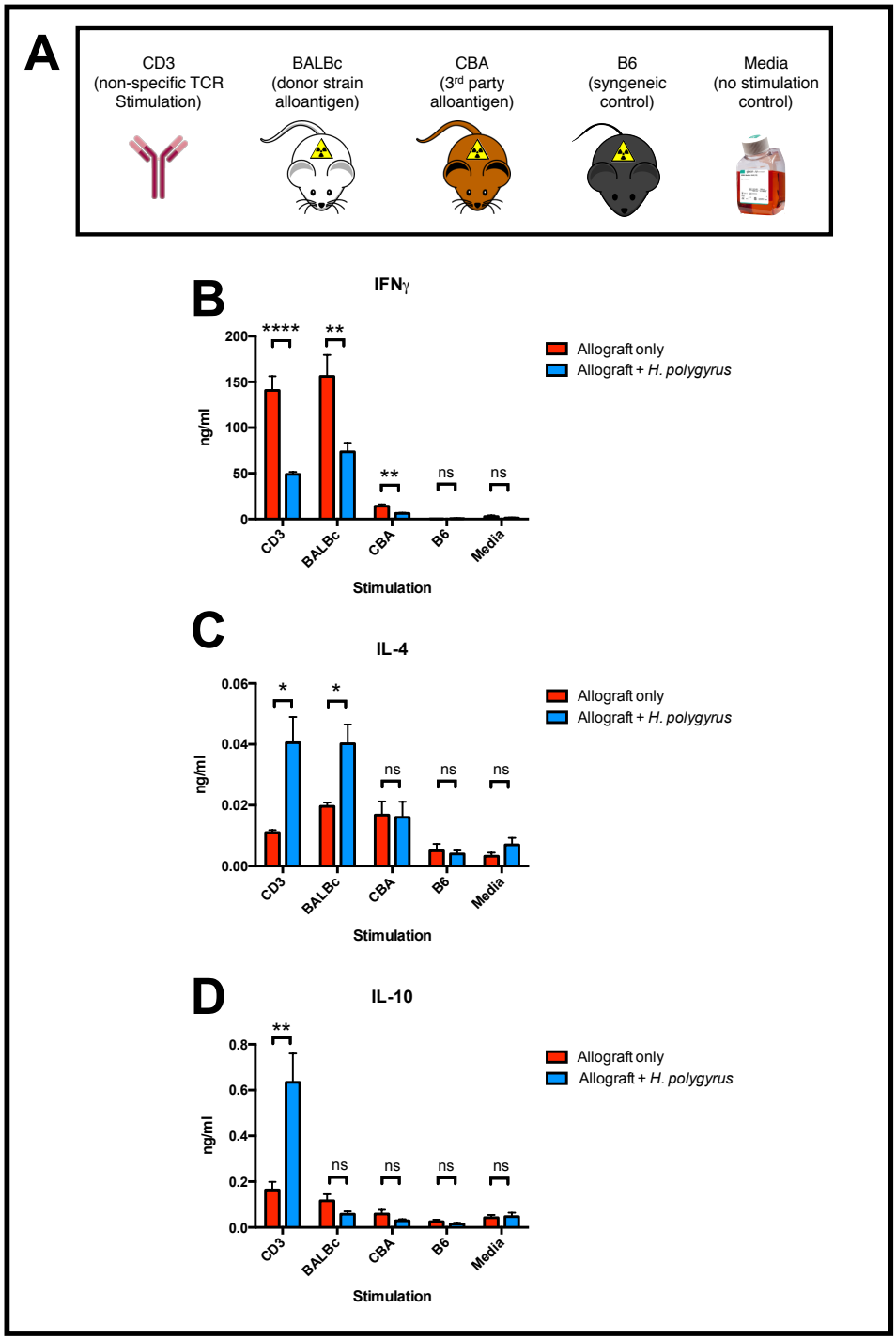


Figure 3-7 *H. polygyrus*-induced Th2 shift is donor alloantigen-specific
(A) As before in figure 3-6, spleens were harvested from C57BL/6 mice 21 days after transplantation of a full-thickness BALB/c skin graft. Allograft recipients either received no treatment (n = 5) or were infected with 200 stage 3 *H. polygyrus* larvae immediately prior to skin grafting (n = 6). Isolated splenocytes (10^6) were restimulated *ex-vivo* for 72 hours at 37°C in duplicate with the following conditions (from left): soluble CD3 (1 μg/ml), 10^6 irradiated BALB/c (donor strain alloantigen) splenocytes, 10^6 irradiated CBA (third party strain alloantigen) splenocytes, 10^6 irradiated C57BL/6 (recipient strain syngeneic antigen) splenocytes, or complete RPMI media. (B) Restimulation supernatant IFN-γ concentration measured by ELISA. (C) Restimulation supernatant IL-4 concentration measured by ELISA. (D) Restimulation supernatant IL-10 concentration measured by ELISA. Data shown are representative of two similar experiments. Statistical significance assessed by multiple unpaired t tests.

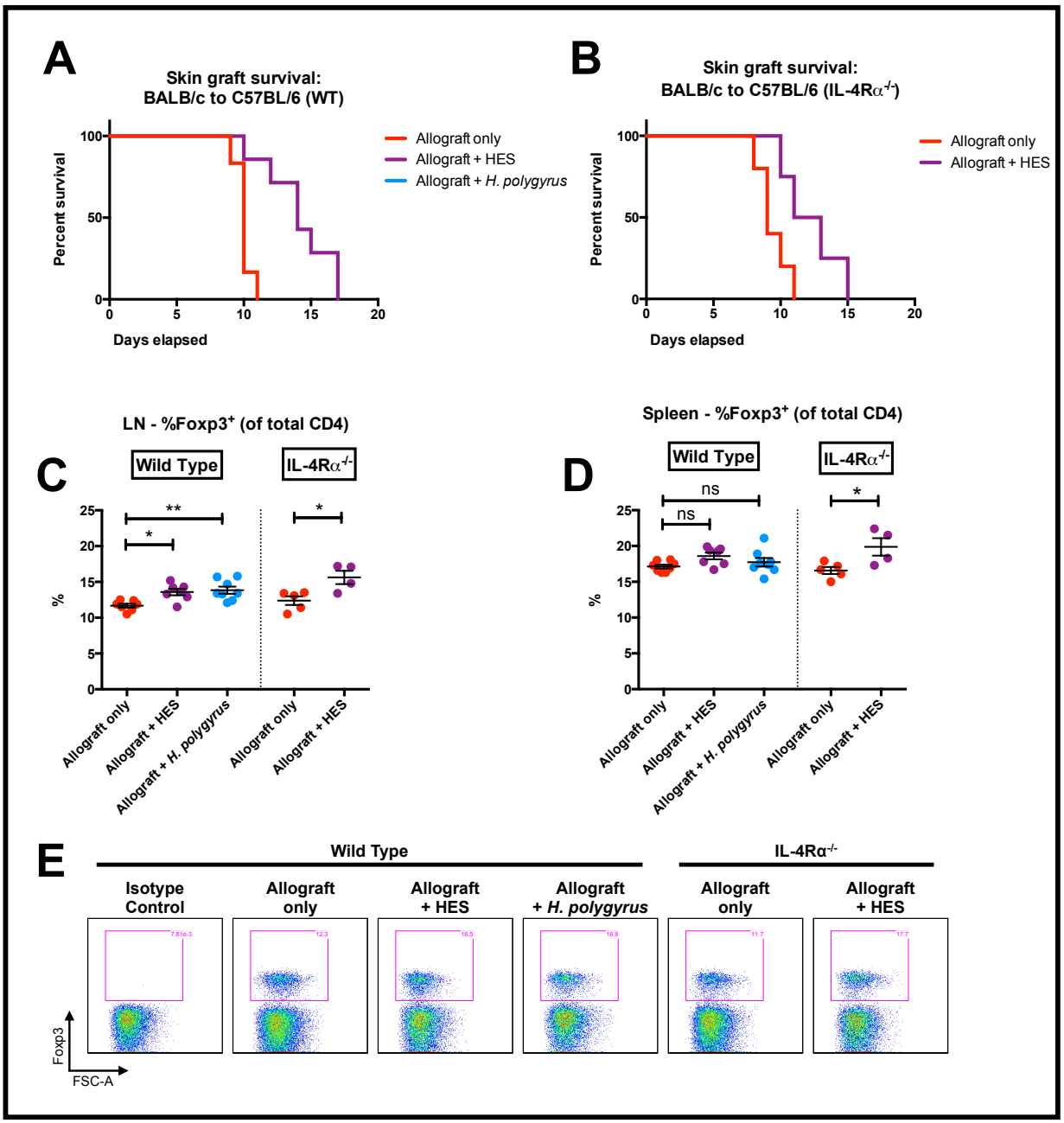


Figure 3-8 HES-mediated allograft protection is not Th2-dependent

(A) Kaplan-Meier curve of full-thickness skin graft survival from BALB/c donor mice to wiild type C57BL/6 recipients, with the following experimental groups: allograft only (n = 6), allograft + HES (skin graft immediately preceded by implantation of an intraperitoneal osmotic minipump eluting 2.6 μg/day of HES; n = 7) and allograft + *H. polygryus* (skin graft with immediately preceded by infection with 200 *H. polygyrus* stage 3 larvae by oral gavage, n = 8). Mantel-Cox comparison of survival curves: allograft vs. allograft + *H. polygyrus*, p = 0.0003; allograft vs. allograft + HES, p = 0.002.

(B) Kaplan-Meier curve of full-thickness skin graft survival from BALB/c donor mice to IL-4 receptor deficient C57BL/6 (IL-4ra$^{-/-}$) recipients, with the following experimental groups: allograft only (n = 5) and allograft + HES (identical administration to (A); n = 4). Mantel-Cox comparison of survival curves: allograft vs. allograft + HES, p = 0.046.

(C) Percentage of CD4^{+} T cells within the allograft draining lymph node expressing Foxp3 21 days after transplantation. (D) Percentage of CD4^{+} T cells within the recipient mouse spleen expressing Foxp3 21 days after transplantation.

(E) Representative flow cytometry plots from (C).

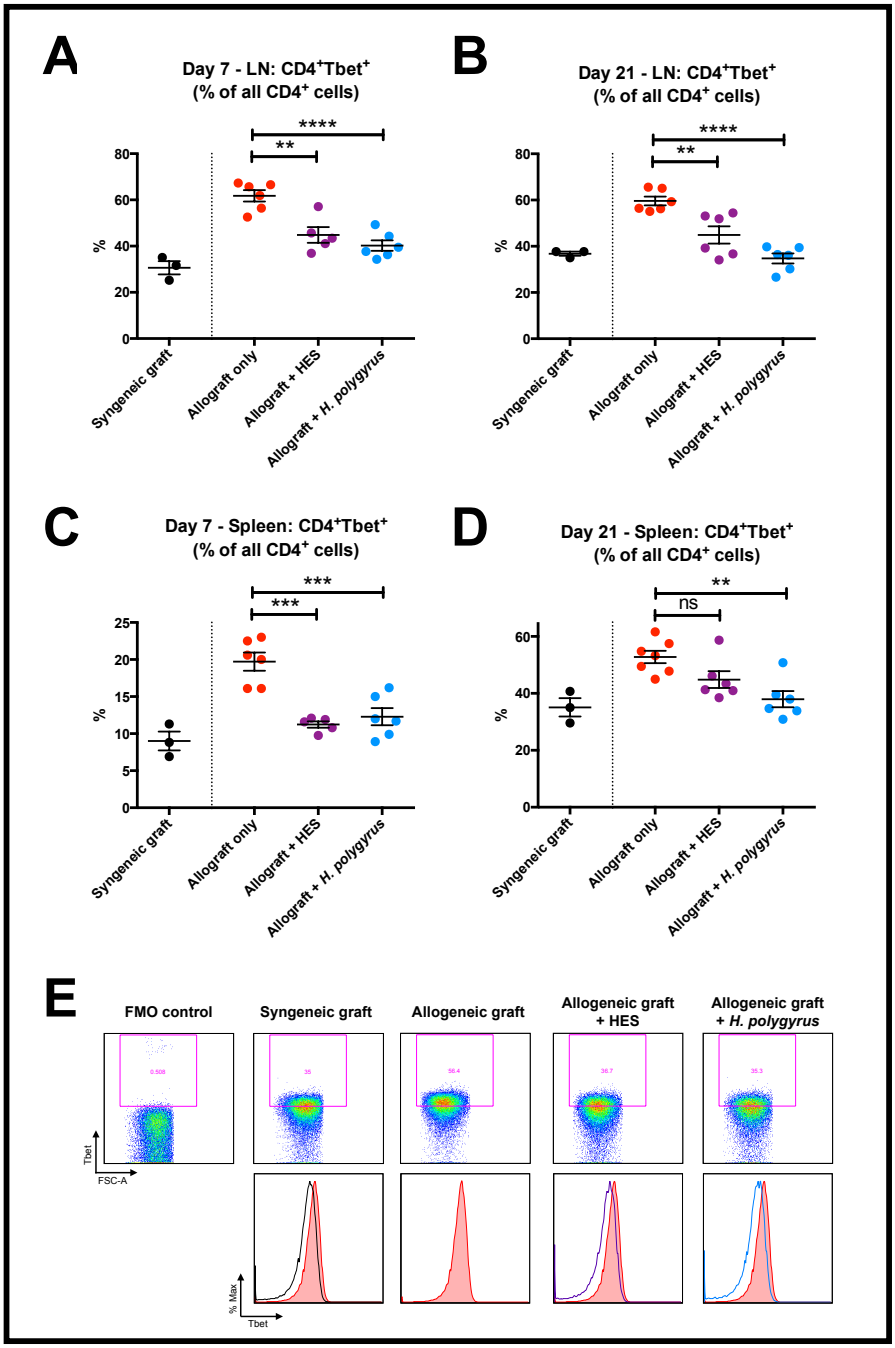


Figure 3-9 *H. polygyrus* and HES suppress expression of Tbet by CD4$^+$T cells
Full-thickness allogeneic (BALB/c to C57BL/6) and syngeneic control (C57BL/6 to C57BL/6) skin grafts were performed with the following experimental conditions: allograft only, allograft + HES (allograft immediately preceded by implantation of an intraperitoneal osmotic minipump eluting 2.6 μg/day of HES) and allograft + *H. polygyrus* (allograft immediately preceded by infection with 200 *H. polygyrus* stage 3 larvae by oral gavage). Two independent experiments were performed to allow for analysis of splenic and allograft draining lymph node CD4$^+$ T cell populations at 7 and 21 days after transplantation. (A) CD4$^+$Tbet$^+$ cells as a percentage of all CD4$^+$ cells within the allograft draining lymph node 7 days after transplantation. (B) CD4$^+$Tbet$^+$ cells as a percentage of all CD4$^+$ cells within the allograft draining lymph node 21 days after transplantation. (C) CD4$^+$Tbet$^+$ cells as a percentage of all splenic CD4$^+$ 7 days after transplantation. (D) CD4$^+$Tbet$^+$ cells as a percentage of all splenic CD4$^+$ 21 days after transplantation. (E) Representative flow cytometry plots and histograms from (B), red shaded area indicates the Tbet expression of the allograft only group.

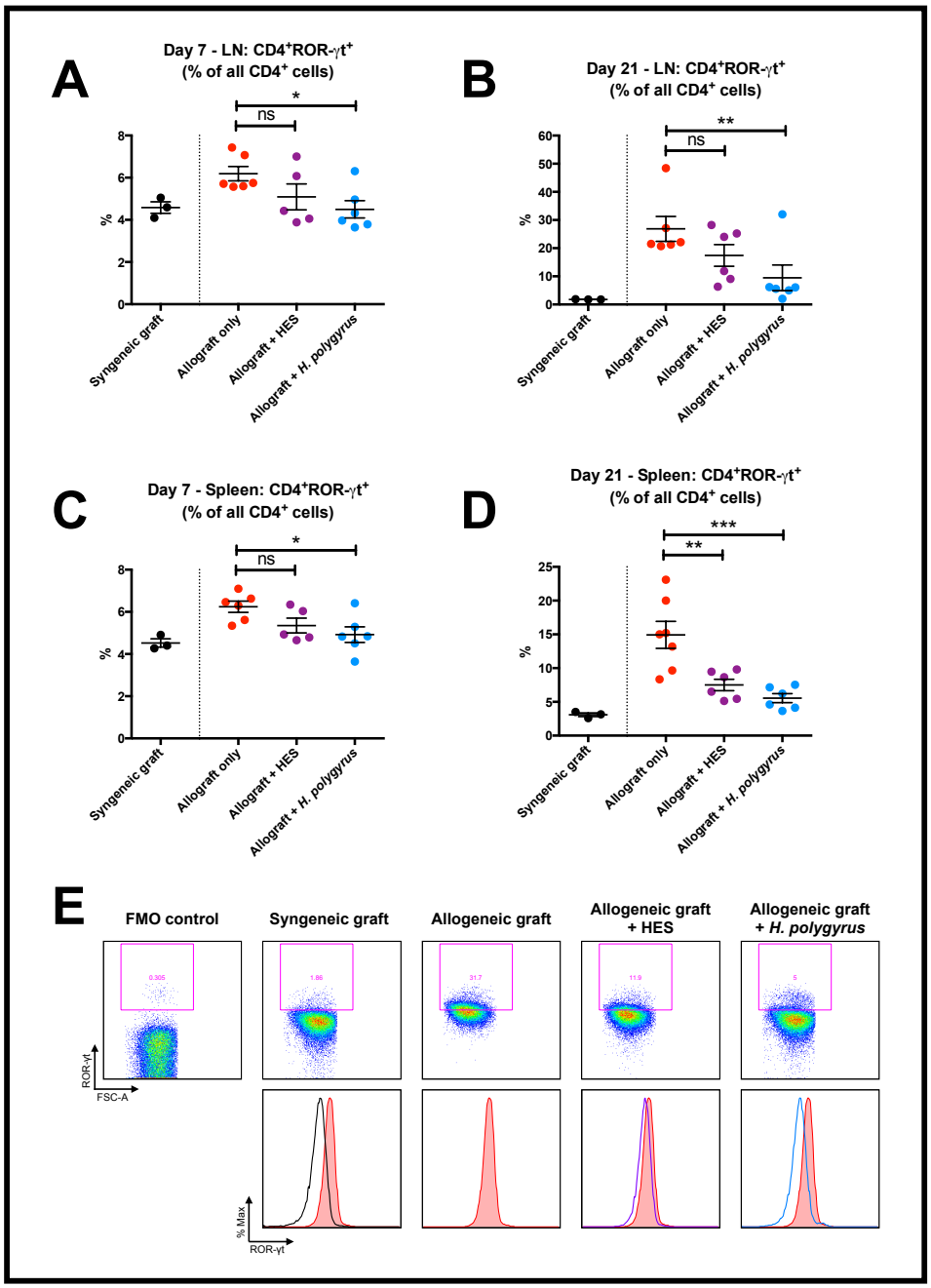


Figure 3-10 *H. polygyrus* infection and HES suppress expression of ROR-γt by CD4$^+$ T cells
Full-thickness allogeneic (BALB/c to C57BL/6) and syngeneic control (C57BL/6 to C57BL/6) skin grafts were performed with the following experimental conditions: allograft only, allograft + HES (allograft immediately preceded by implantation of an intraperitoneal osmotic minipump eluting 2.6 μg/day of HES) and allograft + *H. polygyrus* (allograft immediately preceded by infection with 200 *H. polygyrus* stage 3 larvae by oral gavage). Two independent experiments were performed to allow for analysis of splenic and allograft draining lymph node CD4$^+$ T cell populations at 7 and 21 days after transplantation. (A) CD4$^+$ROR-γt$^+$ cells as a percentage of all CD4$^+$ cells within the allograft draining lymph node 7 days after transplantation. (B) CD4$^+$ROR-γt$^+$ cells as a percentage of all CD4$^+$ cells within the allograft draining lymph node 21 days after transplantation. (C) CD4$^+$ROR-γt$^+$ cells as a percentage of all splenic CD4$^+$ 7 days after transplantation. (D) CD4$^+$ROR-γt$^+$ cells as a percentage of all splenic CD4$^+$ 21 days after transplantation. (E) Representative flow cytometry plots and histograms from (B), red shaded area indicates the ROR-γt expression of the allograft only group.

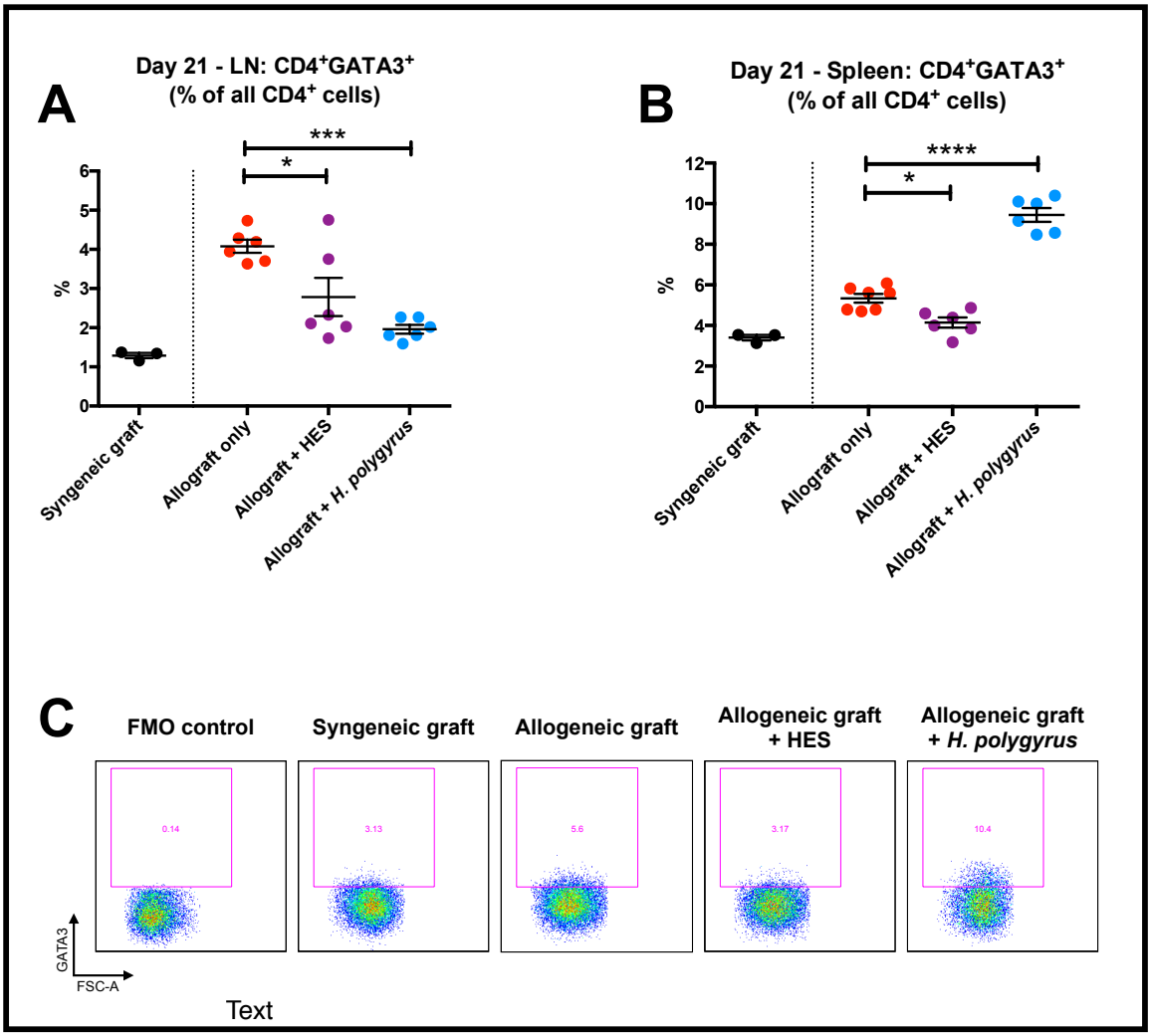


Figure 3-11 ***H. polygyrus*** **infection and HES exert different effects on expression of GATA3 by splenic CD4$^+$ T cells**

(A) CD4$^+$ T cells from the allograft draining (inguinal) lymph node of C57BL/6 mice 21 days after transplantation of a full-thickness BALB/c skin graft or syngeneic C57BL/6 skin graft control. Experimental groups were as follows: allograft only (BALB/c to C57BL/6 skin graft, n = 6), allograft + HES (BALB/c to C57BL/6 allograft immediately preceded by implantation of an intraperitoneal osmotic minipump eluting 2.6 μg/day of HES, n = 6) and allograft + *H. polygyrus* (BALB/c to C57BL/6 skin graft immediately preceded by infection with 200 *H. polygyrus* stage 3 larvae by oral gavage, n = 6). CD4$^+$GATA3$^+$ cells as a percentage of total CD4$^+$ cells shown.

(B) CD4$^+$ T cells from the spleen of the animals described in (A). CD4$^+$GATA3$^+$ cells as a percentage of total CD4$^+$ cells shown.

(C) Representative flow cytometry plots (GATA3$^+$ gating of CD4$^+$ cells shown, from splenic populations in (B)).

Statistical significance assessed by one-way ANOVA with Dunnett's multiple comparisons test. FMO - fluorescence minus one.

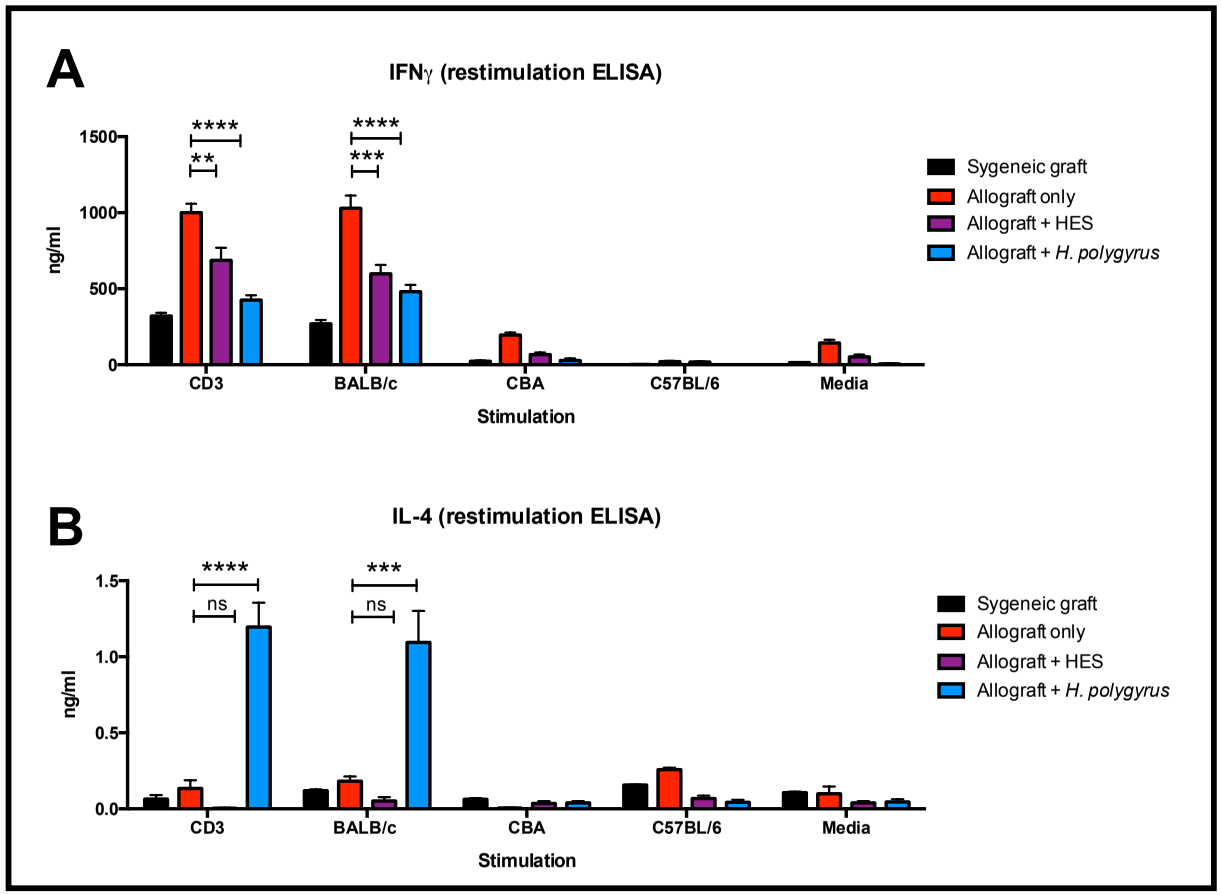


Figure 3-12 *H. polygyrus* infection induces a Th2 shift that is donor alloantigen-specific; HES does not
Splenocytes were harvested from C57BL/6 mice 21 days after transplantation of a full-thickness BALB/c skin graft or syngeneic control graft. Experimental groups were as follows: allograft only, allograft + HES (allograft immediately preceded by implantation of an intraperitoneal osmotic minipump eluting 2.6 μg/day of HES) and allograft + *H. polygyrus* (allograft immediately preceded by infection with 200 *H. polygyrus* stage 3 larvae by oral gavage). Isolated splenocytes (10^6) were restimulated *ex-vivo* for 72 hours at 37°C in duplicate with the following conditions (from left): soluble CD3 (1 μg/ml), 10^6 irradiated BALB/c (donor strain alloantigen) splenocytes, 10^6 irradiated CBA (third party strain alloantigen) splenocytes, 10^6 irradiated C57BL/6 (recipient strain syngeneic antigen) splenocytes, or complete RPMI media. (A) Restimulation supernatant IFNγ concentration measured by ELISA. (B) Restimulation supernatant IL-4 concentration measured by ELISA. Statistical significance assessed by one-way ANOVA with Dunnett's multiple comparisons test.

3.3 Discussion

The results of experiments described in this chapter allow a number of novel conclusions to be drawn. First, treatment with *H. polygyrus* infection can successfully ameliorate allograft rejection. Infection immediately prior to the transplant surgery is sufficient; no period of prophylactic therapy is needed. Secondly, live infection with the parasite is not necessary to achieve allograft protection – treatment with a continuous infusion of HES also improves allograft survival and reduces histological features of rejection compared to allografts without treatment. Thirdly, in the presence of a fully-allogeneic transplant, both *H. polygyrus* and HES promote $Foxp3^+$ Treg differentiation and suppress Th1 and Th17 effector $CD4^+$ T cell phenotype differentiation. Finally, whilst allograft recipients infected with *H. polygyrus* experience a phenotypic switch of alloreactive $CD4^+$ T cells from a Th1 to Th2 subtype, allograft recipients treated with HES do not, and this Th2 response is not necessary for allograft protection.

Allogeneic skin grafts stimulate a particularly aggressive immune response compared to other organ allografts[138,358] and therefore present a significant challenge for tolerance induction therapies to overcome. For example, treatment with exogenous IL-2 complex enables complete tolerance (indefinite survival) of fully-allogeneic islet cell allografts[293] but has no impact on the same strain combination of full-thickness skin grafts (Prof Onur Boyman, personal communication). Functional survival scoring of the BALB/c to C57BL/6 full-thickness skin graft model used for many of the experiments within this chapter may therefore underrepresent the potential benefit of the helminth-derived treatments under investigation. However, the consistently robust alloimmune response generated with this model provides an excellent platform upon which underlying mechanisms of helminth-mediated immunomodulation can be investigated. Likewise, a fully-allogeneic (BALB/c to C57BL/6) transplant combination was employed throughout this chapter so that vigorous alloimmune responses could be generated for further investigation and serial experiments to optimise grafting techniques or treatment conditions could be

performed within a feasible timescale (3-4 weeks). Now that a reliable experimental protocol has been established, further refinement in future experiments with transplants across less severe allogeneic barriers will likely provide greater sensitivity to detect relevant immunomodulatory effects.

As mentioned in section 3.1, full-thickness skin grafts are dependent on a microvascular blood supply and therefore tend to reject in a relatively stochastic manner. The inherent variability in this process is particularly apparent on histological examination at day 7 after transplantation, where individual grafts either resembled normal healthy skin, or were very severely inflamed (seldom between the two extremes) (Figure 3-2). The murine heterotopic cardiac transplantation model (where the left side of a donor heart is anastomosed onto a recipient animal's abdominal aorta and vena cava) is fully-vascularised and therefore grafts undergo a more nuanced rejection process that more closely replicates the situation in human solid organ transplantation. Plans had been made to adopt this technique in Edinburgh following a period of training under the supervision of Mr Gavin Pettigrew at the University of Cambridge; however, quarantine concerns subsequently prevented the presence of *H. polygyrus*-infected animals in the microsurgical facility. Now that a recombinant *H. polygyrus* immunomodulator has been created (TGM, chapter 5), future experiments to assess the impact of TGM in the vascularised allograft model in Cambridge are being planned.

Quantitative comparison of the relative efficacy of HES and live *H. polygyrus* infection in specific immunomodulatory mechanisms (e.g. suppression of Tbet) is difficult because the rate of production, constituent components and location of delivery of secreted products that comprise HES all vary throughout the natural history of an infection. In addition, wild type C57BL/6 mice tend to display significant inter-individual variation in adult worm burdens despite an identical inoculum of stage 3 larvae[354] (Figure 4-3, I). However, it is clear that many of the immunomodulatory actions of *H. polygyrus* can be successfully recapitulated with HES administration, including: expansion of $Foxp3^+$ Treg (Figure 3-8, C+D), suppression of Tbet expression (Figure 3-9), suppression of ROR-γt expression

(Figure 3-10) and suppression of IFN-γ production from alloreactive splenocytes (Figure 3-12).

The observation that *H. polygyrus* infection potently induces GATA3 expression by $CD4^+$ T cells in direct contrast to its suppression by HES (Figure 3-11) is a novel finding. Enhanced IL-4 production upon restimulation with donor allogeneic cells is also specific to allograft recipients treated with *H. polygyrus* infection, indicating that the difference between the Th2 polarising influence of *H. polygyrus* infection and HES is directly affecting the alloreactive T cell population of recipient animals. Preserved allograft protection with HES treatment in IL-4R$\alpha^{-/-}$ mice confirmed that protection afforded by HES is not dependent on a Th2 response (Figure 3-8). Finally, the finding that GATA3 expression by $CD4^+$ T cells is significantly elevated in untreated allograft recipients compared to recipients of syngeneic control grafts (Figure 3-11) might suggest that *H. polygyrus* infection protects transplanted allografts from rejection in spite of inducing a Th2 response rather than because of it.

4 Autoimmunity

4.1 Introduction

The role of helminth infections in ameliorating autoimmune disease has been subject to a much greater degree of investigation than in the field of transplantation. As discussed in section 1.2.3, observational human studies have shown convincing inverse associations between the natural prevalence of helminth infection and autoimmunity[199]. In addition, striking interventional cohort studies performed by Correale *et al*[207,208] demonstrated a significantly lower frequency of clinical relapse and demyelinating lesions in patients with relapsing-remitting MS and concomitant helminth infection compared to a matched uninfected cohort. When helminth infection was cleared pharmacologically, both parameters of disease activity increased to the rate of uninfected patients, suggesting a causal relationship[208]. Preliminary clinical trials of therapeutic infection with live helminth larvae have so far failed to demonstrate a benefit, but they are understandably constrained by safety considerations in their scope and choice of helminth species (as discussed in sections 1.2.5-6). No clinical trials of treatment with helminth-derived immunomodulatory agents have yet been performed.

Of the 14 published studies that have investigated the effects of helminths on EAE (Table 1-1), 5 of these have involved treatment with helminth-derived soluble products (from *S. japonicum*[229], *S. mansoni*[230], *T. spiralis*[226,227], *T. suis*[227], and the schistosome glycan, lacto-N-fructopentaose III (LNFPIII)[228]). Treatment with these soluble mediators was capable of reducing the maximal severity of disease in EAE, but not of reducing the incidence or duration of disease. All have been shown to strongly induce Th2 responses and all except LNFPIII required an extended period of prophylactic administration (2 – 4 weeks) in order to influence the disease course of EAE.

Two studies have been published that investigate the potential for *H. polygyrus* to ameliorate EAE. First, Wilson *et al* demonstrated that adoptive transfer of $CD4^+$ or

$CD19^+$ cells from the mesenteric lymph nodes of *H. polygyrus*-infected mice into C57BL/6 recipients had the effect of suppressing the maximal disease severity of EAE (cells were transferred immediately prior to induction of EAE)[232]. More recently, Donskow-Lysoniewska *et al* have shown that active infection with *H. polygyrus* powerfully suppresses disease and, unlike other helminth species that have been investigated in this context, *H. polygyrus* is capable of treating established disease (precipitating a marked reduction in clinical disease severity within 48 hours of infection)[218]. Investigation of the ability of HES to ameliorate EAE has so far not been reported.

The aims of this chapter are to assess the feasibility of moving from infection with *H. polygyrus* toward treatment with HES and to investigate the underlying mechanisms that might account for any differential therapeutic efficacy between the two.

4.2 Results

4.2.1 *H. polygyrus* infection suppresses experimental autoimmune encephalomyelitis (EAE)

To assess the impact of *H. polygyrus* infection on the development and progression of EAE, comparison was first made between C57BL/6 mice receiving no treatment and an experimental group that were infected with 200 stage 3 *H. polygyrus* larvae by oral gavage. EAE was induced by immunisation with $pMOG_{35-55}$ in complete Freund's adjuvant (CFA) followed by two administrations of pertussis toxin, as outlined in Figure 4-1, A.

Each mouse was assessed according to the classical EAE scoring system described in Table 2-1 and the mean scores for each day are shown in Figure 4-1, B. Daily monitoring revealed an incidence of classical EAE symptoms of 7 out of 11 untreated animals, whilst none of the *H. polygyrus*-infected animals showed any sign of disease (Figure 4-1, C; $p = 0.00047$ as assessed by Fisher's exact test). Analysis of the maximum disease score reached by each animal revealed a mean score of 1.73 (± 0.47) in the untreated group compared to 0 in the *H. polygyrus*-infected group (Figure 4-1, C; $p = 0.0047$ as assessed by the Mann-Whitney U test).

4.2.2 *H. polygyrus* infection suppresses pMOG-specific Th1 and Th17 responses

At the end of the clinical disease course illustrated in Figure 4-1, B (22 days after immunisation), the animals were culled and splenocytes were isolated for *ex-vivo* restimulation with pMOG to assess antigen-specific recall responses. Following suspension in complete RPMI media, 8×10^5 splenocytes were cultured in duplicate for 72 hours at 37 °C with ½log dilutions of $pMOG_{35-55}$, from a maximum concentration of 30 µM. Cytokine concentrations within the culture supernatants were then determined by ELISA.

Upon restimulation, the concentrations of IFNγ (Figure 4-2, A) and IL-17 (Figure 4-2, B) produced by splenocytes from animals that had been infected with *H. polygyrus* were both significantly lower than those from uninfected animals ($p < 0.0001$ for both cytokines, as assessed by 2-way ANOVA). By contrast, the concentration of IL-4 produced by splenocytes from *H. polygyrus*-infected animals was reciprocally increased compared to splenocytes from uninfected controls (Figure 4-2, C). This pattern of an antigen-specific shift from Th1 and Th17 responses towards Th2 is very similar to than seen with donor allogeneic restimulation following skin grafting (Figure 3-6). Although the concentration of IL-4 produced in culture by splenocytes from *H. polygyrus*-infected animals following maximal pMOG restimulation is only 2.67-fold higher than splenocytes from the same animals cultured in media alone, this effect is highly significant when compared to splenocytes from uninfected control animals ($p < 0.0001$, as assessed by two-way ANOVA).

4.2.3 *H. polygyrus*-mediated protection from EAE is mostly Th2-dependent

Following the observation of a pMOG-specific shift towards a Th2 response (Figure 4-2), further experiments were performed in wild-type C57BL/6 mice and IL-4Rα$^{-/-}$ C57BL/6 mice to assess the functional significance of this effect. As before, EAE was induced by immunisation with pMOG in complete Freund's adjuvant (CFA) followed by two administrations of pertussis toxin (outlined in Figure 4-1, A). There were therefore four experimental groups: wild-type C57BL/6 with no treatment (n = 7), wild-type C57BL/6 infected with 200 stage 3 *H. polygyrus* larve (n = 7), IL-4Rα$^{-/-}$ with no treatment (n = 7) and IL-4Rα$^{-/-}$ infected with 200 stage 3 *H. polygyrus* larvae (n = 6).

Each mouse was assessed according to the classical EAE scoring system described in Table 2-1 and the mean scores for each day are shown in Figure 4-3, A (wild-type) and Figure 4-3, B (IL-4Rα$^{-/-}$). Comparison reveals a striking difference: wild-type animals are conferred considerable protection from EAE with *H. polygyrus* infection

(evidenced by a delayed onset and reduction in maximal disease severity), whilst the effect of *H. polygyrus* infection on IL-4Rα$^{-/-}$ animals is very much diminished, with a small delay in disease onset only.

The incidence of symptomatic disease (score > 0) in wild-type animals was 6/7 with no treatment compared to 2/7 with *H. polygyrus* infection (Figure 4-3, C; p = 0.051), whilst the incidence in IL-4Rα$^{-/-}$ animals with no treatment was 6/7 with no treatment and 4/6 with *H. polygyrus* infection (Figure 4-3, D; p = 0.437).

The mean maximum disease score of wild-type untreated animals was 4.7-fold greater than that of wild-type mice treated with *H. polygyrus* infection (Figure 4-3, E; p = 0.026), whilst the mean maximum score of treated and untreated IL-4Rα$^{-/-}$ mice showed no significant difference (Figure 4-3, F). The duration of symptomatic disease was not significantly reduced with *H. polygyrus* infection in wild-type mice or IL-4Rα$^{-/-}$ mice (Figure 4-3, G+H).

The worm burden (number of adult worms within the duodenum and proximal jejunum) for each mouse was also assessed and while average numbers did not differ significantly, the range was found to be much greater in the wild-type animals (39 - 172) compared to IL-4Rα$^{-/-}$ (98 – 115).

Finally, the proportions of Foxp3^{+} Treg within the splenic CD4^{+} T cell population were assessed and no significant difference was seen at this late time point (26 days after immunisation) (Figure 4-3, J).

4.2.4 *H. polygyrus* infection induces ST2 expression by Foxp3^{-} effector CD4^{+} T cells via a Th2-dependent mechanism

At the end of the EAE disease course described in section 4.2.3 (Figure 4-3, A+B), splenocytes were isolated from wild-type C57BL/6 and IL-4Rα$^{-/-}$ mice for further analysis by flow cytometry.

H. polygyrus infection induced a 5-fold increase in expression of the transcription factor GATA3 in wild-type mice (Figure 4-4, A+C; $p < 0.0001$), but this effect was diminished in IL-4Rα$^{-/-}$ mice (to a 1.57-fold increase in expression, $p = 0.011$). MFI of GATA3 expression of all CD4^{+} cells showed the same relationship (Figure 4-4, B), indicated the impaired ability of IL-4Rα$^{-/-}$ mice to mount an effective Th2 response to *H. polygyrus* infection.

Expression of the IL-33 receptor, ST2, was also assessed and found to be significantly increased in response to *H. polygyrus* infection in wild type mice, but not IL-4Rα$^{-/-}$ (Figure 4-4, D+F), indicating a Th2-dependent mechanism. ST2 expression by Foxp3^{+} Treg was unaffected by *H. polygyrus* infection in wild-type and IL-4Rα$^{-/-}$ mice (Figure 4-4, E+F).

4.2.5 *H. polygyrus* infection induces PD-1 expression by Foxp3^{-} effector CD4^{+} T cells via a Th2-independent mechanism

The splenic CD4^{+} T cell populations described in 4.2.4 (from wild-type C57BL/6 and IL-4Rα$^{-/-}$ mice 26 days after induction of EAE) were also assessed for expression of Programmed Cell Death Protein 1 (PD-1).

H. polygyrus infection was found to significantly increase expression of PD-1 by Foxp3^{-} effector CD4^{+} T cells in wild-type C57BL/6 mice and to an even greater extent in IL-4Rα$^{-/-}$ mice (1.4-fold and 1.84-fold increases, respectively), indicating a

Th2-independent mechanism (Figure 4-5, A+C). As with ST2, PD-1 expression by Foxp3$^+$ Treg was unaffected by *H. polygyrus* infection in wild-type and IL-4Rα$^{-/-}$ mice (Figure 4-5, B+C).

4.2.6 HES delays the onset of EAE, but is less effective than *H. polygyrus* infection in suppressing disease

In order to deconstruct the effects of HES from the physical presence of *H. polygyrus* with live infection, C57BL/6 mice were divided into two experimental groups: one receiving an intraperitoneal osmotic minipump eluting 2.64 μg of HES per day and the other undergoing a laparotomy without minipump insertion (sham surgery) to control for the possible effect of a cortisol stress response. After 24 hours, both groups were subjected to the same EAE induction protocol as before (immunisation with pMOG and CFA, followed by two injections of pertussis toxin, as described in section 2.5). At this stage, the maximum duration of HES administration was limited to 14 days, due to a relative ceiling in the concentration of HES that could be achieved by ultrafiltration (as described in section 2.3.3).

The animals were assessed on a daily basis according to the classical EAE scoring system described in Table 2-1 and the mean scores for each day are shown in Figure 4-6, A. The morphology of the EAE curve for animals treated with HES via minipump bears a very close resemblance to that of IL-4Rα$^{-/-}$ mice treated with *H. polygyrus* infection. The incidences of symptomatic disease and maximal disease severity scores were not significantly different between experimental groups (Figure 4-6, B+C). However, treatment with HES did significantly delay the onset of disease (by a mean of 2.15 days, $p = 0.0049$; Figure 4-6, D) and therefore also reduce the total duration of symptomatic disease (Figure 4-6, E).

The proportions of Foxp3$^+$ Treg within the splenic CD4$^+$ T cell population were assessed and no significant difference was seen at this late time point (23 days after immunisation) (Figure 4-6, F).

4.2.7 HES promotes expression of PD-1 and ST2 by $CD4^+$ $Foxp3^-$ effector T cell populations

At the end of the EAE disease course described in section 4.2.6 (Figure 4-6, A), splenocytes were isolated from HES-treated and untreated mice for further analysis by flow cytometry.

Analysis of the $CD4^+$ T cell population revealed a 2.71-fold increase in PD-1 expression by $Foxp3^-$ effector cells ($p = 0.0003$; Figure 4-7, A+C). PD-1 expression by $Foxp3^+$ Treg was not significantly changed (Figure 4-7, B). Expression of ST2 followed the same pattern, with a 4.02-fold increase within the $Foxp3^-$ effector cell population ($p < 0.0001$; Figure 4-7, D+F), but no significant change within the Treg population (Figure 4-7, E).

4.2.8 HES suppresses colitis following adoptive transfer of naïve $CD4^+Foxp3^-$ T cells into $RAG1^{-/-}$ recipients

Another established model of T cell-mediated immunopathology is the T cell transfer model of colitis, which facilitates multiple functional outcome measures and objective histological assessment of pathology[359]. To assess the impact of HES in ameliorating colitis, $RAG1^{-/-}$ mice were divided into two experimental groups – receiving either no treatment, or a continuous infusion of HES via an intraperitoneal osmotic minipump eluting 2.64 μg/day for 14 days (maximum possible duration of HES administration was determined by the concentration that could be achieved). One day after minipump surgery (day 0), naïve $CD4^+$ T cells were isolated from the spleen and peripheral lymph nodes of Foxp3-GFP reporter mice by MACS $CD4^+$ positive selection followed by FACS, gating: $CD4^+CD25^-GFP^-$. Following sorting, 5×10^5 cells were adoptively transferred IV into each $RAG1^{-/-}$ mouse. Recipient $RAG1^{-/-}$ mice were monitored on a regular basis and treatment with HES was shown to ameliorate disease as evidenced by maintenance of mean body weight more than 21 days after T cell transfer (Figure 4-8, A), a 0.42-fold change in mean pathology

scores (p = 0.04; Figure 4-8, B) and consistently lower mean Disease Activity Index scores over time (Figure 4-8, C).

4.2.9 HES induces Foxp3 expression in adoptively transferred antigen-specific T cell populations

For a more focussed analysis of the underlying mechanisms through which *H. polygyrus* infection and HES can modulate immune responses that are relevant to EAE and autoimmunity in general, the effects exerted on an adoptively transferred antigen-specific $CD4^+$ T cell population were assessed.

$CD4^+$ T cells from DO11.10 transgenic mice (BALB/c background) are characterised by a T cell receptor that specifically recognises ovalbumin (OVA) peptide antigen that is traceable by flow cytometry (cells are KJ1-26^+). For this experiment, $CD4^+$ T cells were isolated from the spleens of female DO11.10 mice by MACS positive selection and then further purified for naïve T cells by FACS (gating on $CD4^+$KJ1-$26^+CD25^-CD62L^{hi}$). Recipient female wild-type BALB/c mice were divided into three experimental groups: sham surgery (laparotomy without minipump insertion), implantation of an intraperitoneal osmotic minipump eluting 2.64 μg of HES per day, or infection with 200 *H. polygyrus* stage 3 larvae by oral gavage. 10^6 Naïve DO11.10 $CD4^+$ T cells were adoptively transferred into recipient mice by intravenous injection 24 hours after surgery or infection. After a further 24 hours, recipient mice were immunised with $pOVA_{323-339}$ in CFA by bilateral subcutaneous hind limb injections (in an identical fashion to immunisation with pMOG for induction of EAE). Recipient mice were culled five days after immunisation for analysis of splenic and inguinal lymph node $CD4^+$ T cell populations by flow cytometry (experimental design outlined in Figure 4-9, A).

The total number of DO11.10 cells recovered from the inguinal lymph node of HES-treated animals was significantly reduced compared to untreated controls (0.25-fold change, p = 0.015; Figure 4-9, B), whilst animals infected with *H. polygyrus* showed the same trend, but the difference was not statistically significant (0.5-fold change, p

= 0.141, Figure 4-9,B). The number of DO11.10 cells recovered from the spleens of recipient animals was not significantly changed following treatment with HES or *H. polygyrus* infection.

The proportion of Foxp3$^+$ Treg as a percentage of the transferred DO11.10 CD4$^+$ T cell population was significantly increased following treatment with HES (1.82-fold change, p = 0.036, Figure 4-9, C). Again, animals treated with *H. polgygrus* showed the same trend, but the difference was not statistically significant (1.45-fold change, p = 0.273; Figure 4-9, C). The Foxp3$^+$ Treg percentage of recovered DO11.10 CD4$^+$ T cell from the spleens of recipient animals was not significantly changed by either treatment (Figure 4-9, D).

4.2.10 *H. polygyrus* infection and HES suppress proliferation of Foxp3$^-$ effector CD4$^+$ T cells in adoptively transferred antigen-specific T cell populations

The adoptively transferred DO11.10 CD4$^+$ T cell population described in section 4.2.9 was further analysed for cellular proliferation by staining for the nuclear protein, Ki67.

Proliferation of Foxp3$^+$ Treg within the transferred cell population recovered from the inguinal lymph nodes and spleen was not significantly changed by treatment with HES or *H. polgyurus* infection (Figure 4-10, A+B).

By contrast, treatment with HES suppressed proliferation (as assessed by Ki67 expression) of Foxp3$^-$ effector CD4$^+$ T cells within the transferred cell population, both those recovered from the inguinal lymph node (0.59-fold change, p = 0.028; Figure 4-10, C+E) and from the spleen (0.75-fold change, p = 0.043). *H. polygyrus* infection showed an almost identical effect, with suppression of Ki67 expression by transferred CD4$^+$Foxp3$^-$ cells in both the inguinal lymph node (0.61-fold change, p = 0.042, Figure 4-10, C+E) and the spleen (0.61-fold change, p = 0.007; Figure 4-10, D).

4.2.11 Identification and recombinant production of the TGF-β mimic within HES

Since the confirmation of TGF-β activity within HES[233], much attention has focussed on the identification of the component molecule mediating this effect. Given the considerable number of proteins and other molecules within HES, and the fact that none has any resemblance of TGF-β, a reductionist approach was required. As shown in Figure 4-11, the components of HES were separated by charge and size fractionation so that individual fractions could be assessed for TGF-β activity (with the MFB-F11 bioassay, described in Figure 5-1). One fraction from each technique was found to be active and 23 common component molecules were subsequently identified. Each of these was then optimised for expression in a HEK293 cell line with pSecTag2 vectors. At the end of this process (November 2014), one molecule, named the TGF-β mimic (TGM), was found have a near-perfect correlation with abundance on fractionation and TGF-β bioassay activity. TGM has a predicted molecular weight of 48.9 kDa, it bears little sequence homology with any other known peptide and it forms the basis of Chapter 5.

4.2.12 Transcriptome microarray analysis of *in vivo* stimulated antigen-specific $CD4^+$ T cells

In assessing the impact of TGM on autoimmunity, high-throughput analysis of gene expression in response to treatment was adopted as a strategy to identify the broadest possible spectrum of immunomodulatory mechanisms that are potentially invoked by this protein and to guide the direction of further investigation. Microarray analysis also presented the potential to identify key differences between the mechanisms of action behind treatment with *H. polygyrus* infection, HES and TGM.

Whilst *H. polygyrus* and HES are known to act on several innate and adaptive immune cell populations, this experiment focussed specifically on $CD4^+$ T cells so that sufficient resolution could be achieved to aid the functional interpretation of the changes in gene expression observed. For the same reason, analysis of an antigen-specific $CD4^+$ T cell population was chosen. This did dramatically reduce the

number of cells that could be recovered for RNA extraction and analysis, but offered the potential benefit of considerably improved precision; changes in gene expression observed within this cell population were directly relevant mechanistically.

In light of these considerations, the following experiment was performed (Figure 4-12): treatment of wild-type BALB/c recipient mice in four experimental groups: intraperitoneal PBS-eluting minipump (control), intraperitoneal HES-eluting minipump (2.6 μg/day), intraperitoneal TGM-eluting minipump (79.2 ng/day) and inoculation with 200 L3 *H. polygyrus* by oral gavage (n = 5 per group; treatment conditions initiated on day -2). On day -1, antigen-specific $CD4^+$ T cells were isolated from the spleens of female DO11.10 ($KJ1\text{-}26^+$) mice by MACS positive selection and then further purified by FACS (gating $CD4^+KJ1\text{-}26^+CD25^-CD62L^{hi}$). 10^6 cells were then adoptively transferred (IV) into each of the wild-type BALB/c mice that had commenced treatment the previous day. On day 0, each mouse was immunised with 20 μg pOVA (the cognate antigen for DO11.10 cells) and CFA containing 50 μg heat-killed M. tuberculosis. Five days after immunisation, the animals were culled and the transferred DO11.10 $CD4^+$ T cell population was recovered by FACS (gating $CD4^+KJ1\text{-}26^+$). Cells were sorted into RNAlater and snap frozen on dry ice.

Preparation of RNA for microarray analysis was performed by Dr Bob Holt (Hologic Ltd). RNA was prepared (amplified, fragmented, labelled and hybridised) with an Affymetrix GeneChip® Pico kit (according to the manufacturer's instructions). Following assessment of RNA quality after amplification, the optimal 3 samples (of 5) from each experimental group was selected for analysis on an Affymetrix GeneChip® Mouse Transcriptome Pico Assay 1.0.

The Affymetrix Pico Assay is specifically designed for use with small cell samples and to therefore function effectively with as little as 100 pg of total RNA (as few as 10 cells). Each of the biological replicates for this experiment was comprised of more than 10^4 cells, yet technical issues relating to low RNA yields affecting the array were reported and the subsequent gene expression data produced unfortunately exhibits such a degree of variability as to preclude meaningful comparison. A

summary of gene expression comparisons from this experiment is included in appendix B; raw p values are shown, but when corrected for the number of comparisons made, none of the changes in gene expression reported are statistically significant. Plans to repeat this experiment are underway.

Fortunately, other approaches to characterise TGM-mediated immunomodulation and its functional significance have been much more successful, as discussed in chapter 5.

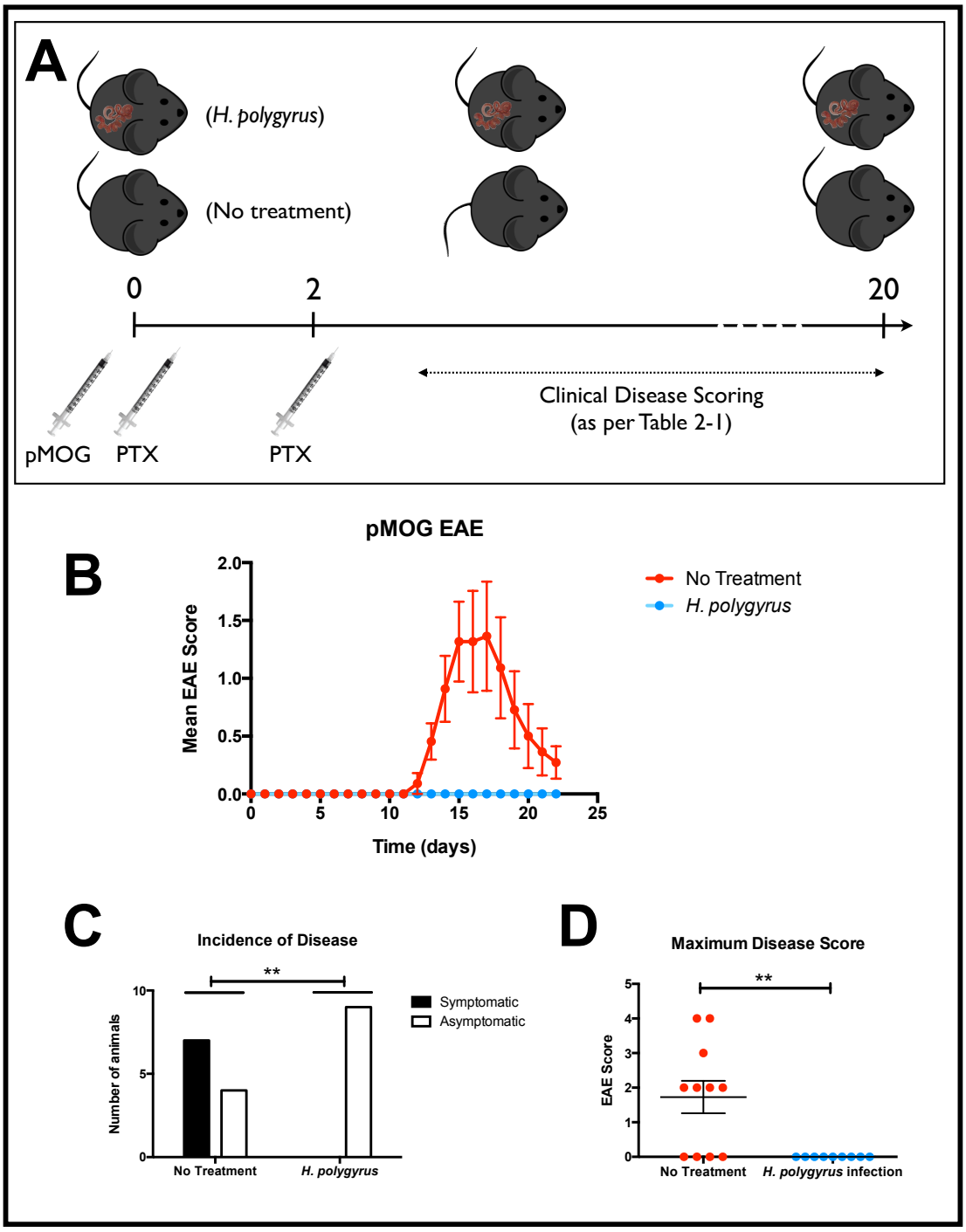


Figure 4-1 *H. polygyrus* infection suppresses experimental autoimmune encephalomyelitis (EAE)

(A) Experimental design: C57BL/6 mice were immunised wtih 100 μg $pMOG_{35-55}$ and Complete Freund's Adjuvant containing 50 μg heat-killed *M. tuberculosis* via bilateral hind limb subcutaneous injection. Pertussis toxin (PTX, 200ng in 500 μl) was administered by intraperitoneal injection at the time of immunization and again 48 hours later. Animals were separated into two experimental groups - one received no treatment and the other was infected with 200 *H. polygyrus* stage 3 larvae by oral gavage immediately prior to immunisation. Animals were monitored on a daily basis and accorded a score for clinical progression of disease (as per Table 2-1). (B) Mean clinical EAE disease scores over time (scoring system: Table 2-1). (C) Incidence of clinically symptomatic disease (i.e. animals with a score > 0). (D) Maximum disease score achieved by each animal at any point over the study period.

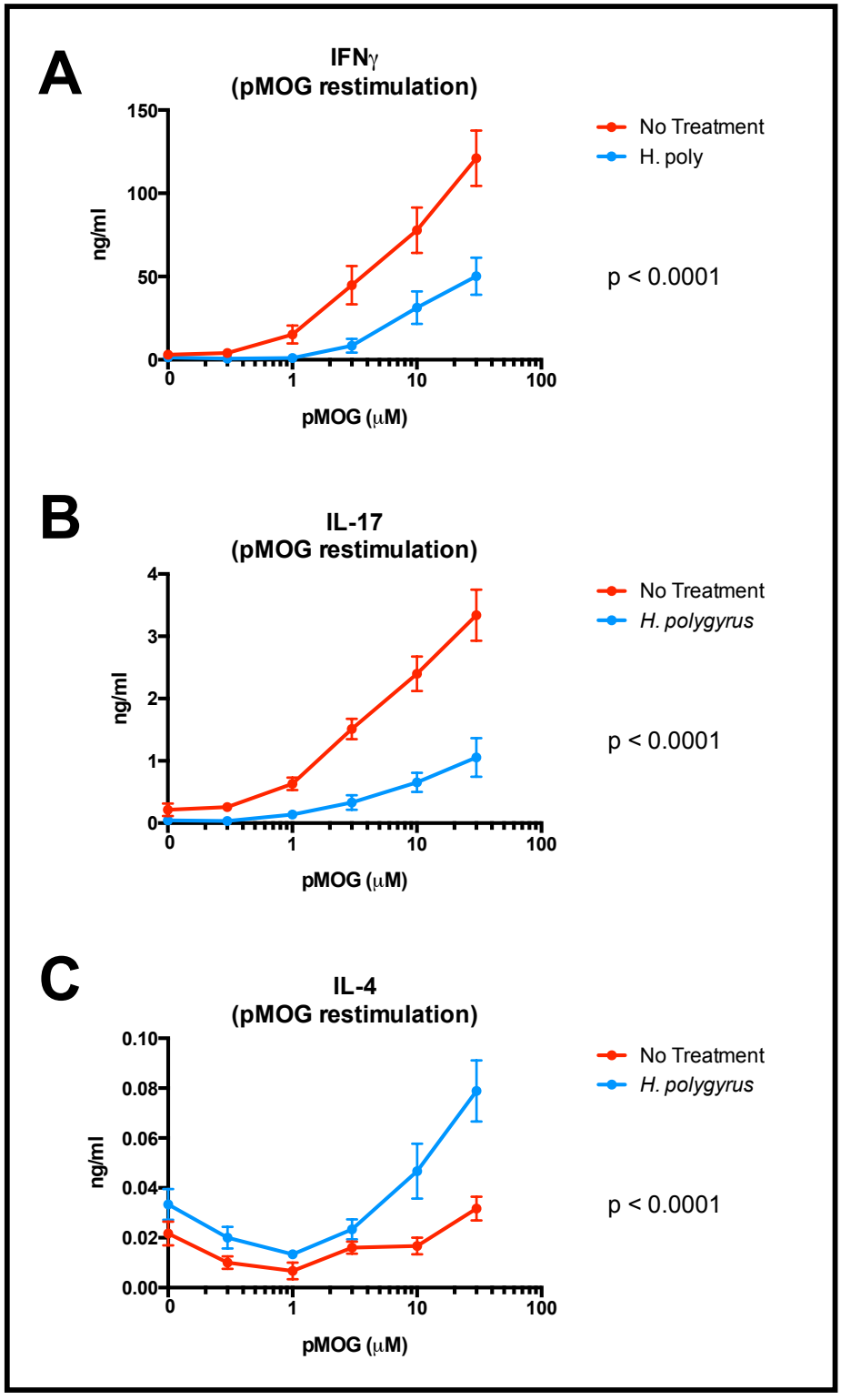


Figure 4-2 *H. polygyrus* infection suppresses pMOG-specific Th1 and Th17 responses

At the end of the disease course shown in Figure 4-1, B (22 days after immunisation), splenocytes were isolated from the two experimental groups of animals - pMOG EAE with no treatment (n = 11) and pMOG EAE with simultaneous *H. polygyrus* infection (n = 9). Isolated splenocytes (8 x 10^5) were restimulated *ex-vivo* for 72 hours at 37°C in duplicate with variable concentrations of pMOG suspended in complete RPMI media as shown. (A) Restimulation supernatant IFNγ concentration measured by ELISA. (B) Restimulation supernatant IL-17 concentration measured by ELISA. (C) Restimulation supernatant IL-4 concentration measured by ELISA.

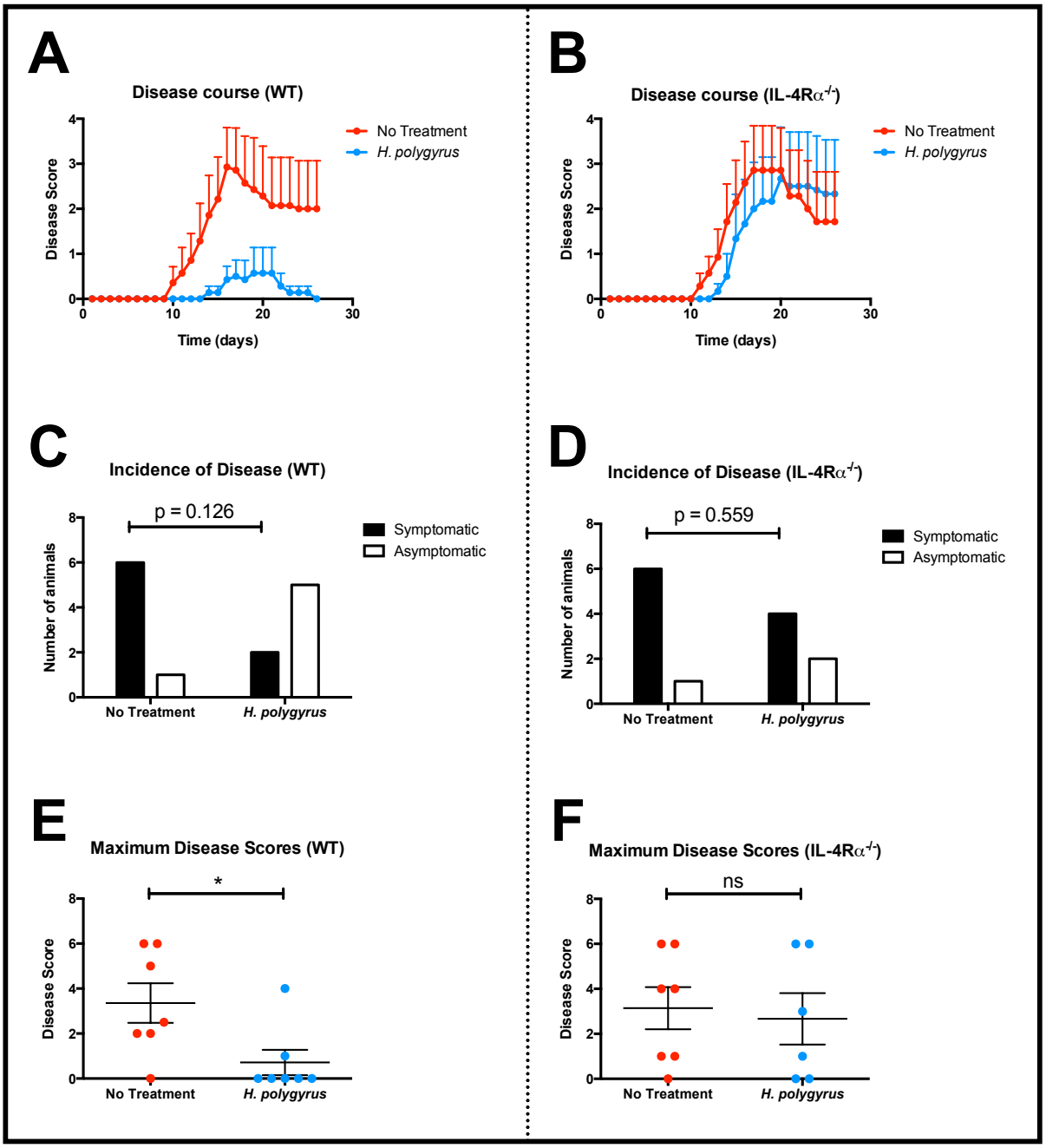


Figure 4-3 *H. polygyrus*-mediated protection from EAE is partially Th2-dependent
Experimental design: pMOG EAE was established as before (Figure 4-1, A), but in parallel groups of wild type C57BL/6 and IL-4 receptor deficient (IL-4Rα$^{-/-}$) C57BL/6 mice. Animals received either no treatment (WT: n = 7; IL-4Rα$^{-/-}$: n = 7) or were infected with 200 *H. polygyrus* stage 3 larvae by oral gavage immediately prior to immunisation (WT: n = 7; IL-4Rα$^{-/-}$: n = 6). Animals were monitored on a daily basis and accorded a score for clinical progression of disease (as per Table 2-1). (A and B) Mean clinical EAE disease scores over time (scoring system: Table 2-1). (C and D) Incidence of clinically symptomatic disease (i.e. animals with a score > 0). (E and F) Maximum disease score achieved by each animal at any point over the study period.

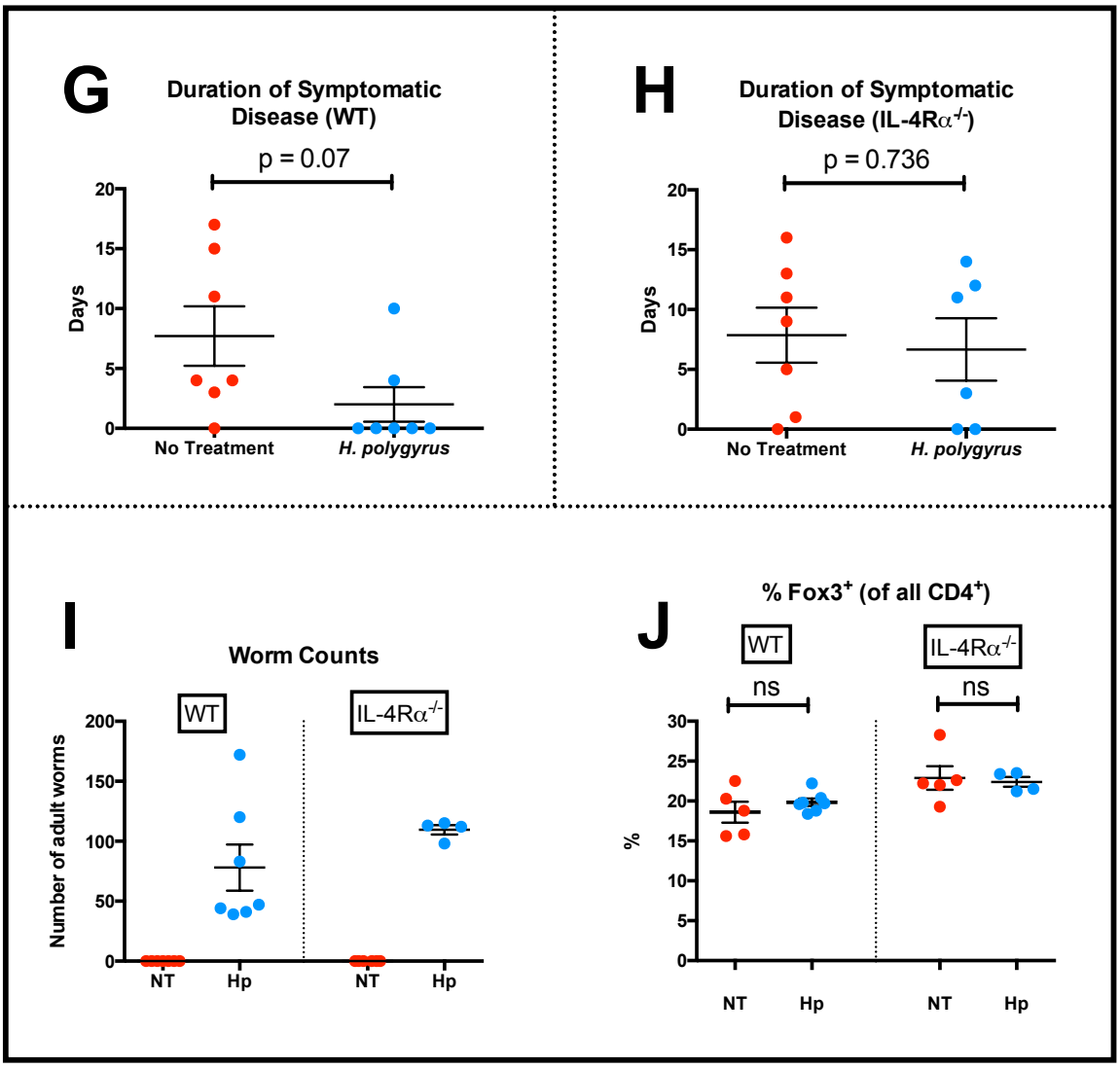


Figure 4-3 [Continued]
(G and H) Duration of symptomatic disease (from onset until recovery or the end of the study period). (I) Worm burden as assessed by the number of adult worms retrieved from the duodenum and jejunum at the end of the study period. (J) Percentage Foxp3$^+$ Treg of all CD4$^+$ T cells resident in the spleen (representative flow cytometry plots shown in Figure 4-4, F).

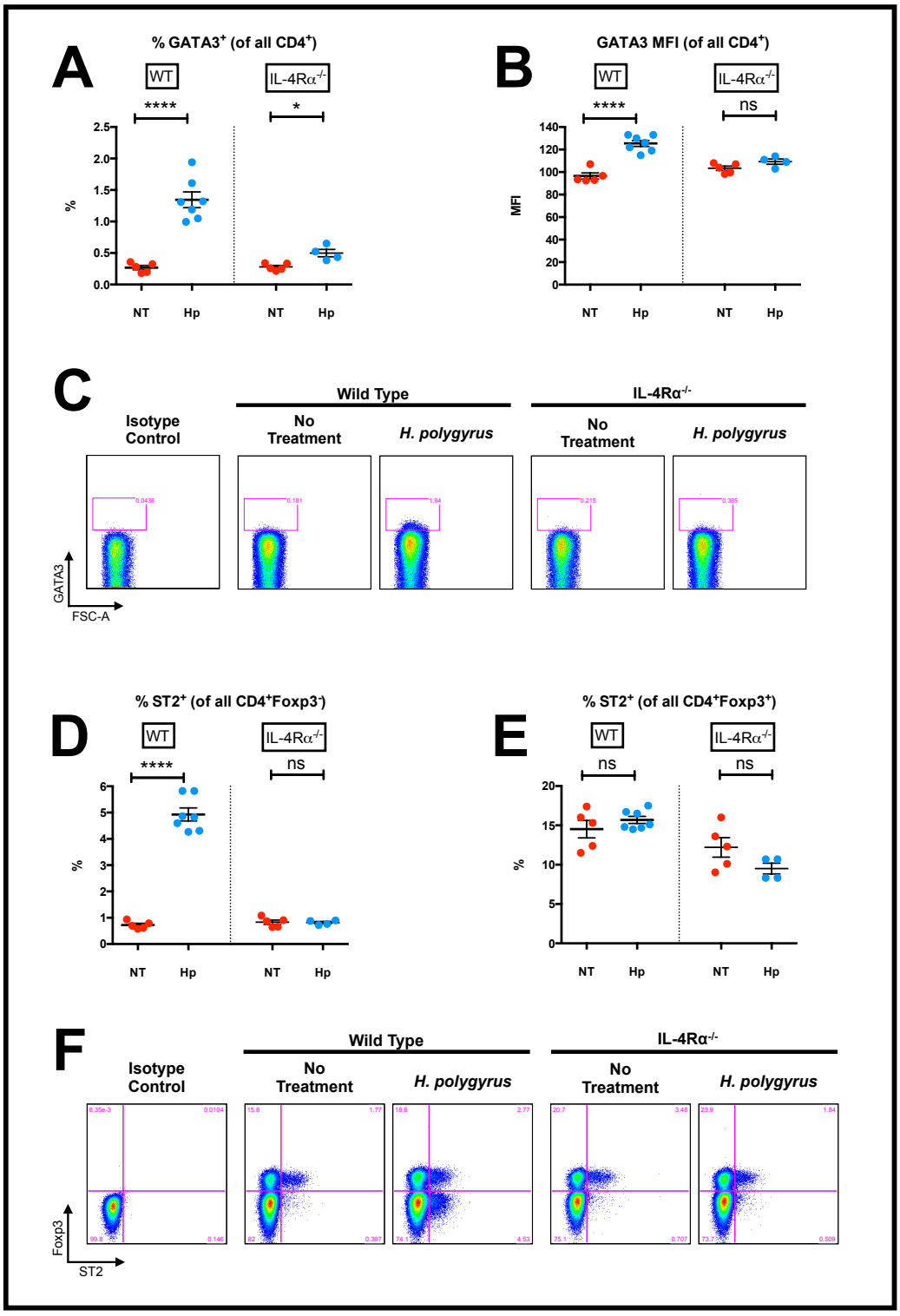


Figure 4-4 *H. polygyrus* infection induces ST2 expression by Foxp3$^-$ effector CD4$^+$ T cells via a Th2-dependent mechanism

Flow cytometric analysis of CD4$^+$ T cells isolated from the spleens of wild type C57BL/6 and IL-4Rα$^{-/-}$ mice with either no treatment or *H. polygyrus* infection immediately prior to immunisation with pMOG to initiate EAE (Figure 4-3). Splenocytes were isolated 26 days after immunisation.

(A) Percentage GATA3 expression (of all splenic CD4$^+$ cells). (B) MFI of GATA3 expression (of all splenic CD4$^+$ cells). (C) Representative flow cytometry plots of (A). (D) Percentage ST2 expression (of all splenic CD4$^+$Foxp3$^-$ cells). (E) Percentage ST2 expression (of all splenic CD4$^+$Foxp3$^+$ cells). (F) Representative flow cytometry plots of (D).

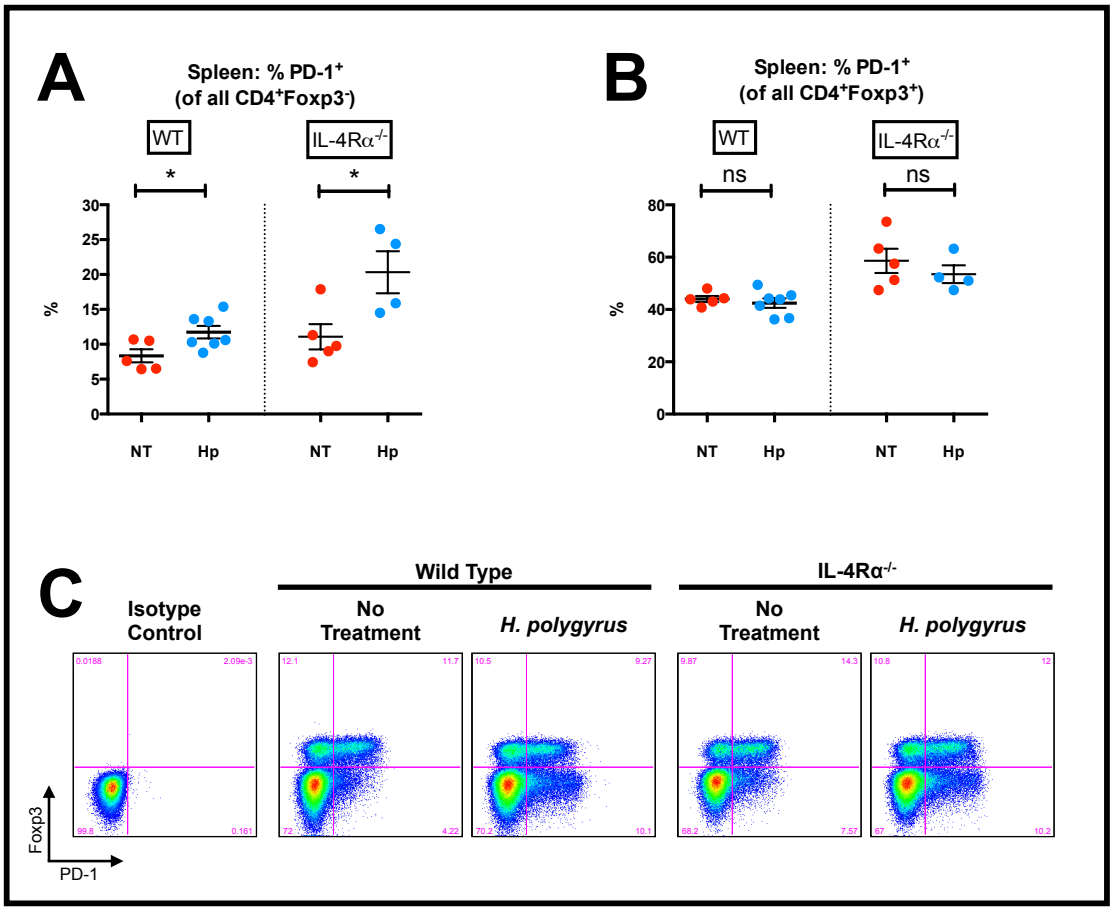


Figure 4-5 *H. polygyrus* infection induces PD-1 expression by Foxp3$^-$ effector CD4$^+$ T cells via a Th2-independent mechanism

Flow cytometric analysis of CD4$^+$ T cell populations isolated from the spleens of mice from the experiment described in Figure 4-3 (pMOG EAE in parallel groups of wild type C57BL/6 and IL-4Rα$^{-/-}$ C57BL/6 mice, with animals receiving either no treatment, or infection with 200 *H. polygyrus* stage 3 larvae by oral gavage immediately prior to immunisation).

(A) PD-1 expression (% of CD4$^+$Foxp3$^-$ cells). (B) PD-1 expression (% of CD4$^+$Foxp3$^+$ cells). (C) Representative flow cytometry plots from (A).

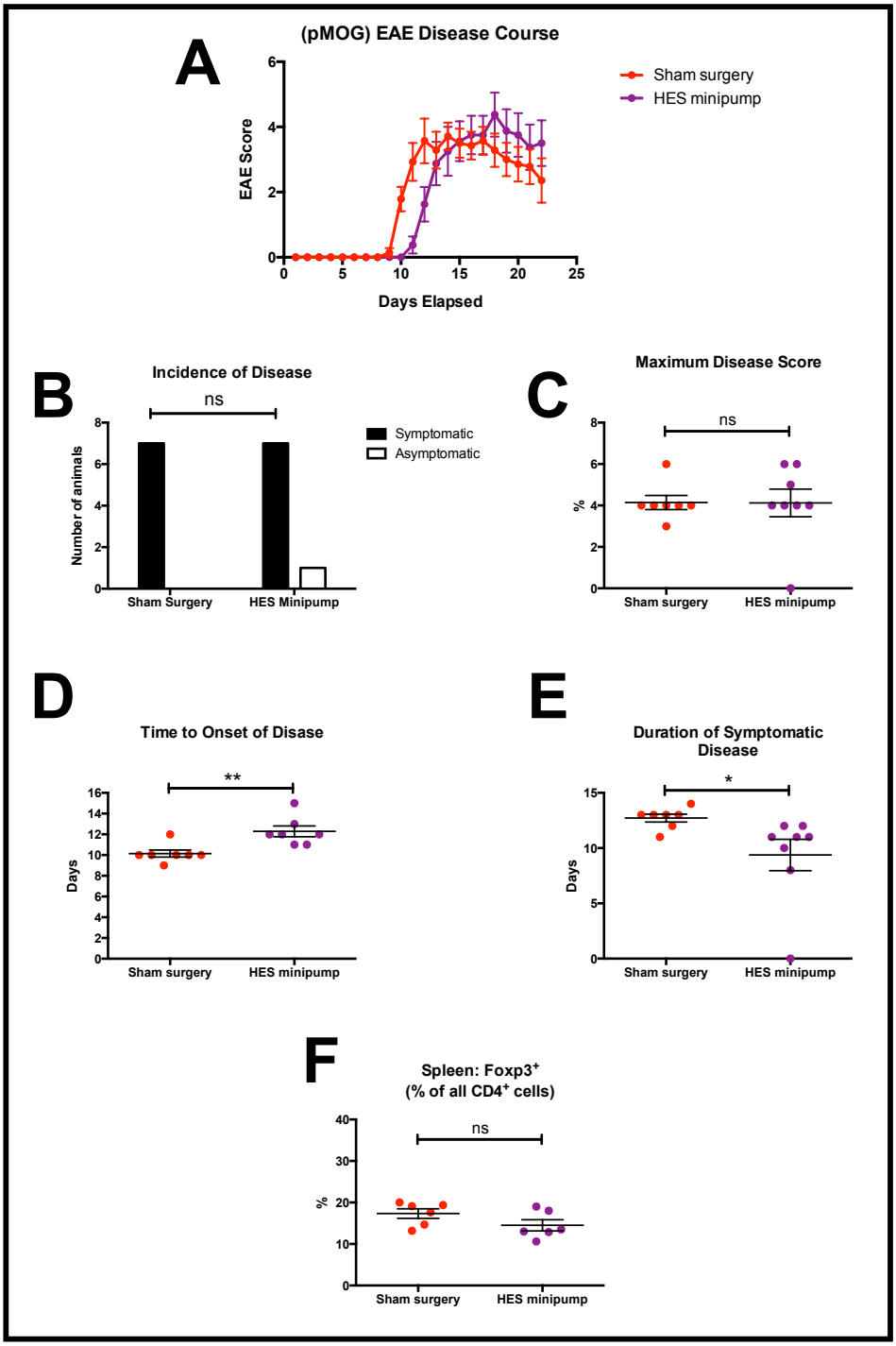


Figure 4-6 HES delays the onset of EAE, but is less effective than *H. polygyrus* infection in suppressing disease

Experimental design: C57BL/6 mice were immunised with 100 μg pMOG and Complete Freund's Adjuvant containing 50 μg heat-killed *M. tuberculosis* via bilateral hind limb subcutaneous injection. Pertussis toxin (PTX, 200ng in 500 μl) was administered by intraperitoneal injection at the time of immunistation and again 48 hours later. Animals were separated into two experimental groups - one received an intraperitoneal osmotic minipump eluting 2.6 μg/day of HES for 14 days, the other underwent an identical laparotomy with no minipump as a sham surgery control. Surgery was performed one day prior to immunisation (day -1). Animals were monitored on a daily basis and accorded a score for clinical progression of disease (as per Table 2-1). (A) Mean clinical EAE disease scores over time (scoring system: Table 2-1). (B) Incidence of clinically symptomatic disease (i.e. animals with a score > 0). (C) Maximum disease score achieved by each animal at any point over the study period. (D) Time until onset of symptomatic disease. (E) Duration of symptomatic disease. (F) Percentage Foxp3$^+$ Treg of all CD4$^+$ T cells resident in the spleen (representative flow cytomtetry plots shown in Figure 4-7, F).

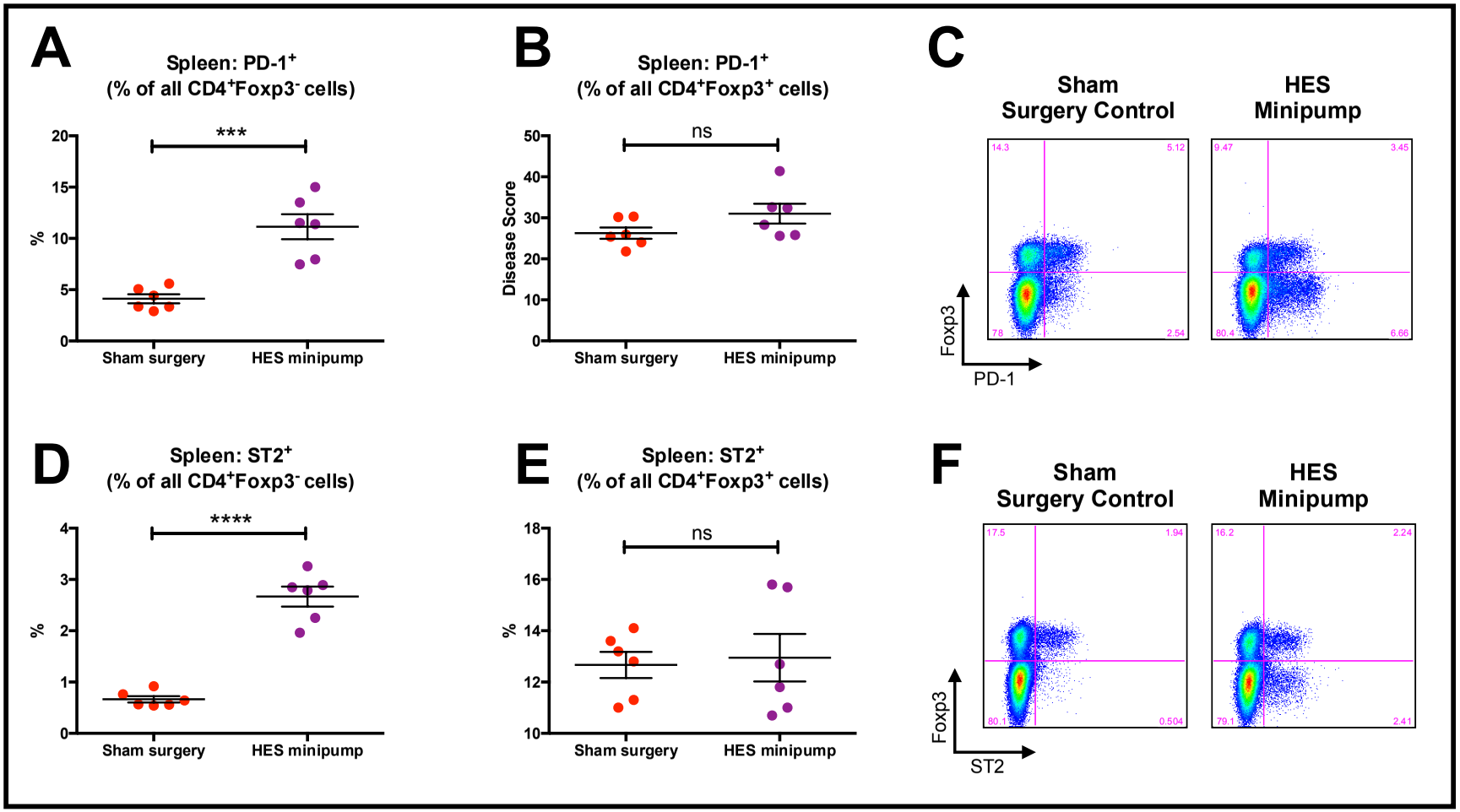


Figure 4-7 HES promotes expression of PD-1 and ST2 by CD4$^+$Foxp3$^-$ effector T cell populations

Flow cytometric analysis of CD4$^+$ T cell populations isolated from the spleens of mice from the experiment described in Figure 4-6 (pMOG EAE, with animals receiving either an intraperitoneal osmotic minipump eluting 2.6 μg/day of HES for 14 days, or undergoing an identical laparotomy with no minipump as a sham surgery control.).

(A) PD-1 expression (% of CD4$^+$Foxp3$^-$ cells). (B) PD-1 expression (% of CD4$^+$Foxp3$^+$ cells). (C) Representative flow cytometry plots from (A).

(D) ST2 expression (% of CD4$^+$Foxp3$^-$ cells). (E) ST2 expression (% of CD4$^+$Foxp3$^+$ cells). (D) Representative flow cytometry plots from (A).

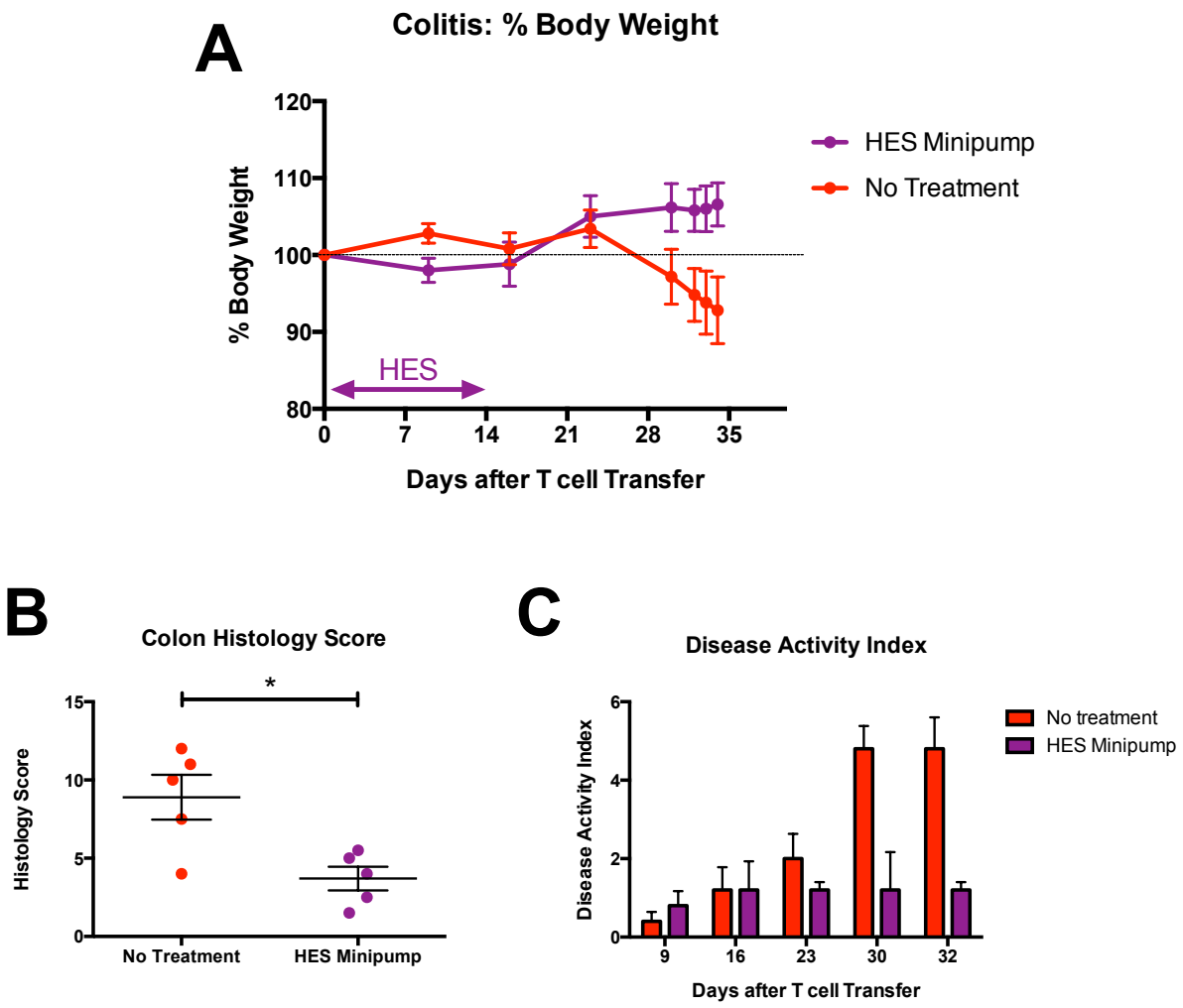


Figure 4-8 HES suppresses colitis following adoptive transfer of naive $CD4^{+}Foxp3^{-}$ T cells into $RAG1^{-/-}$ recipients

Experimental design: naive effector $CD4^{+}$ T cells were isolated from the spleen and peripheral lymph nodes of Foxp3-GFP reporter mice by positive MACS selection for $CD4^{+}$, followed by FACS, gating: $CD4^{+}CD25^{-}GFP^{-}$. Following isolation, $5x10^{5}$ T cells were adoptively transferred into $RAG1^{-/-}$ recipients. Recipient mice received either no treatment or a 14-day infusion of HES via an intraperitoneal minipump (eluting 2.6 μg/day of HES). Animals were monitored regularly throughout the experiment and colonic specimens were then prepared for histological assessment.

(A) Percentage change from body weight at the start of the experimental period.

(B) Colon histology scores, according to the scoring system described in Section 2.6.2

(C) Progression of Disease Activity Index scores over time (as described in Section 2.6.3).

Colitis experiments were performed jointly with Dr Danielle Smyth; histology scoring was performed by Dr Mark Arends.

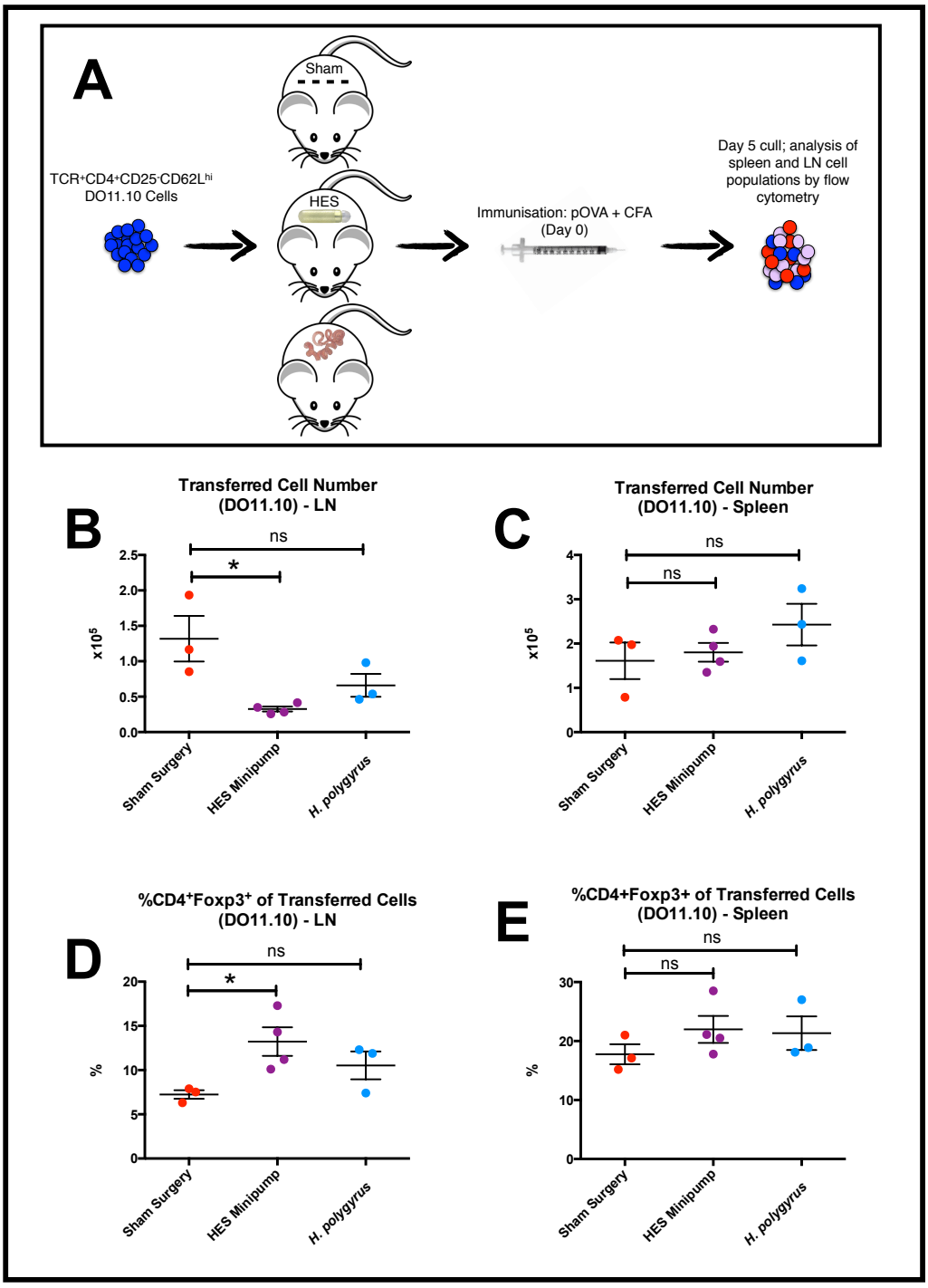


Figure 4-9 HES induces Foxp3 expression in adoptively transferred antigen-specific T cell populations

(A) Experimental design. Three experimental groups; Day -2: inoculation with 200 L3 *H. polygyrus* by oral gavage, insertion of intraperitoneal HES-eluting minipump and sham laparotomy control. Day -1: adoptive transfer (IV) of 10^6 naive $CD4^+$ DO11.10 T cells. Day 0: immunisation with 20 μg pOVA and CFA containing 50 μg heat-killed *M. tuberculosis*. Day 5: cull and preparation of single cell suspensions from spleen and inguinal lymph nodes for analysis by flow cytometry.

(B) Number of adoptively transferred cells detected on day 5 detected in inguinal LNs and (C) spleen.

(D) Percentage of $CD4^+Foxp3^+$ cells from the transferred cell population in inguinal LNs and (E) spleen.

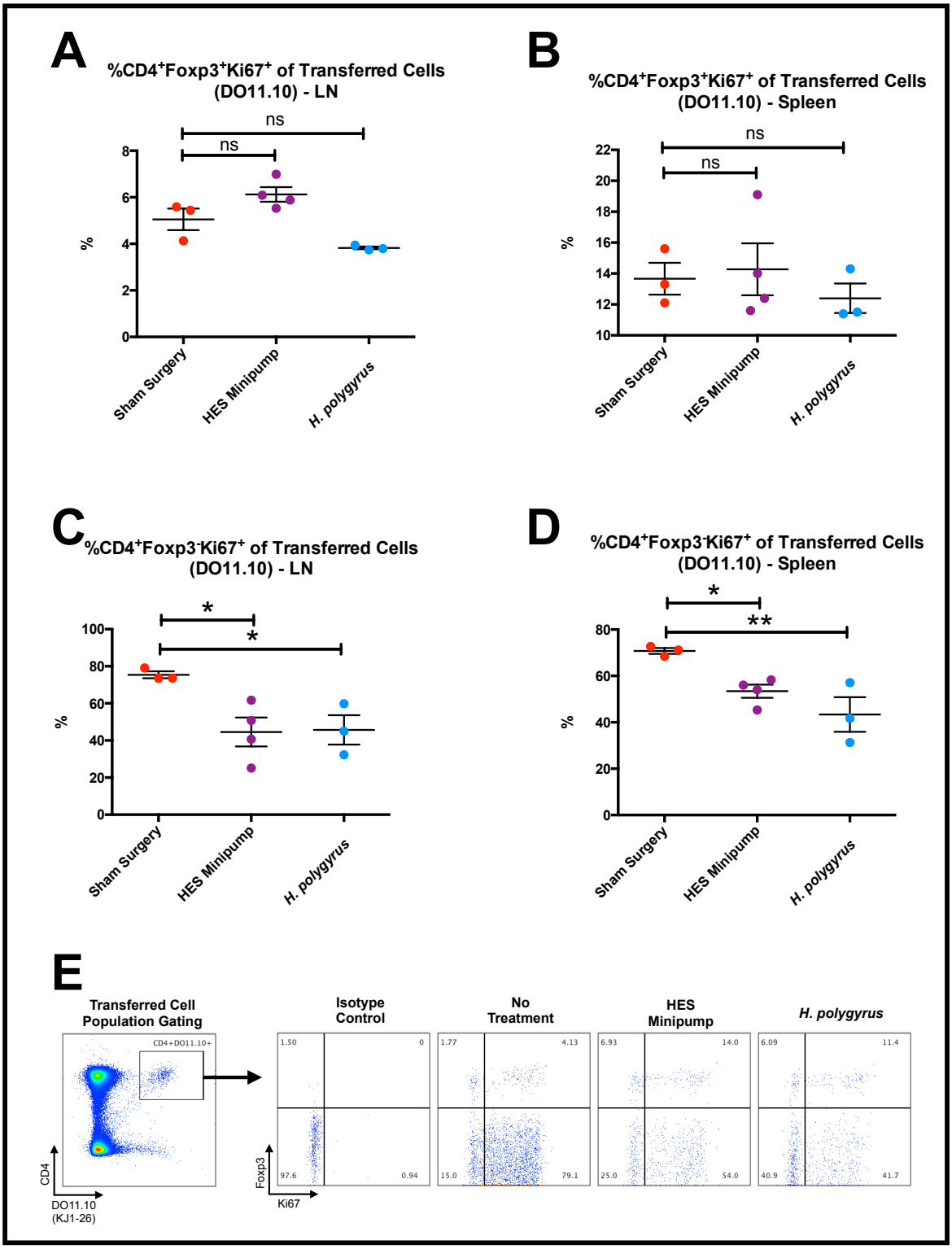


Figure 4-10 *H. polygyrus* infection and HES suppress proliferation of Foxp3$^-$ effector CD4$^+$ T cells in adoptively transferred antigen-specific T cell populations

(A) CD4$^+$Foxp3$^+$ (Treg) percentage of total DO11.10 transferred cell population in inguinal lymph nodes and (B) spleen.

(C) Ki67$^+$ percentage of DO11.10 Treg population in inguinal lymph nodes and (D) spleen.

(E) Representative flow cytometry plots from (A-D).

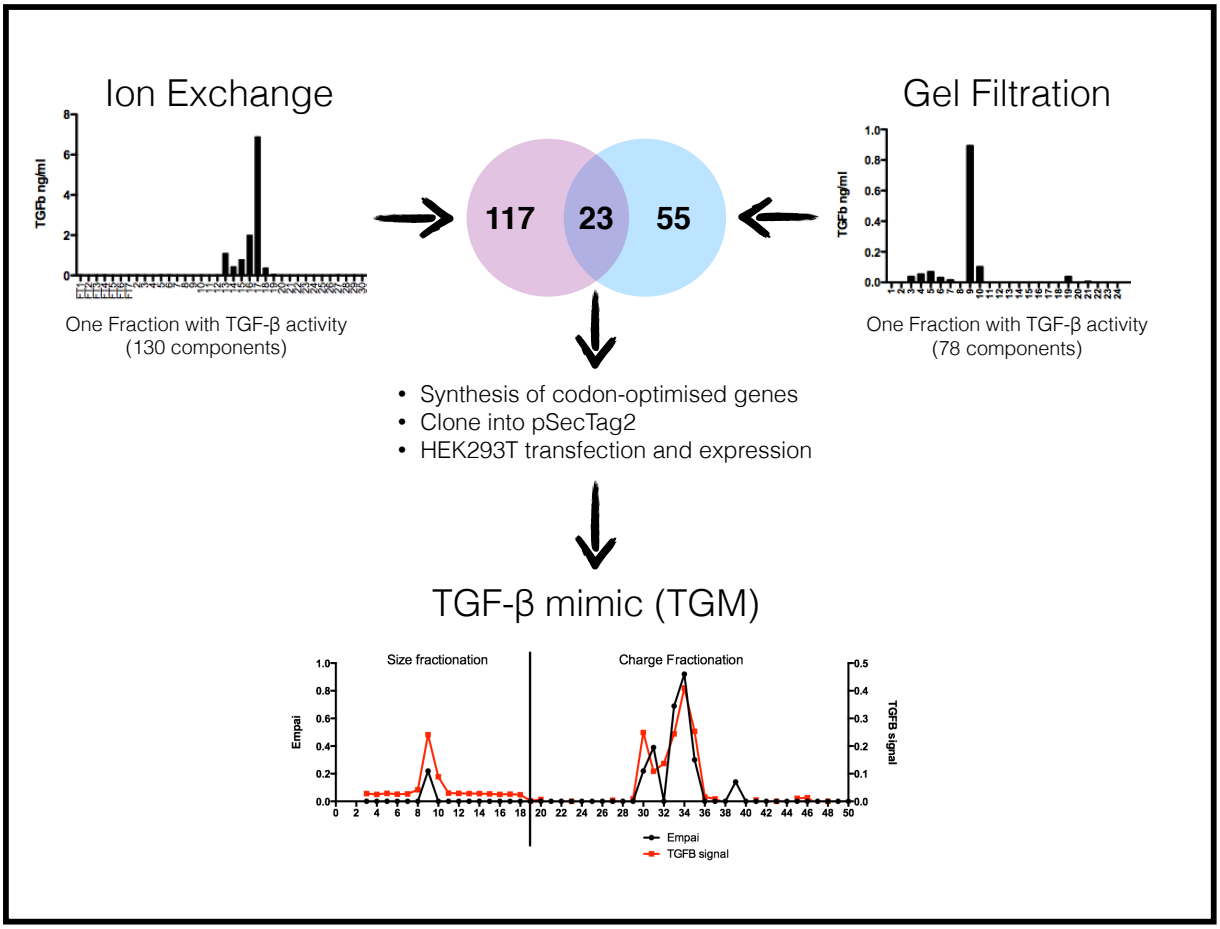


Figure 4-11 Identification and recombinant production of the TGF-β mimic with HES (adapted from McSorley & Maizels)
Summary of experimental approach (Kara Fibley, Henry McSorley & Rick Maizels) culminating in the discovery of the TGF-β-like molecule within HES, named the TGF-β mimic (TGM), in November 2014.
TGF-β activity assessed by culture with the MFB-F11 reporter cell line (described in Figure 5-1). Empai - exponentially modified protein abundance index.

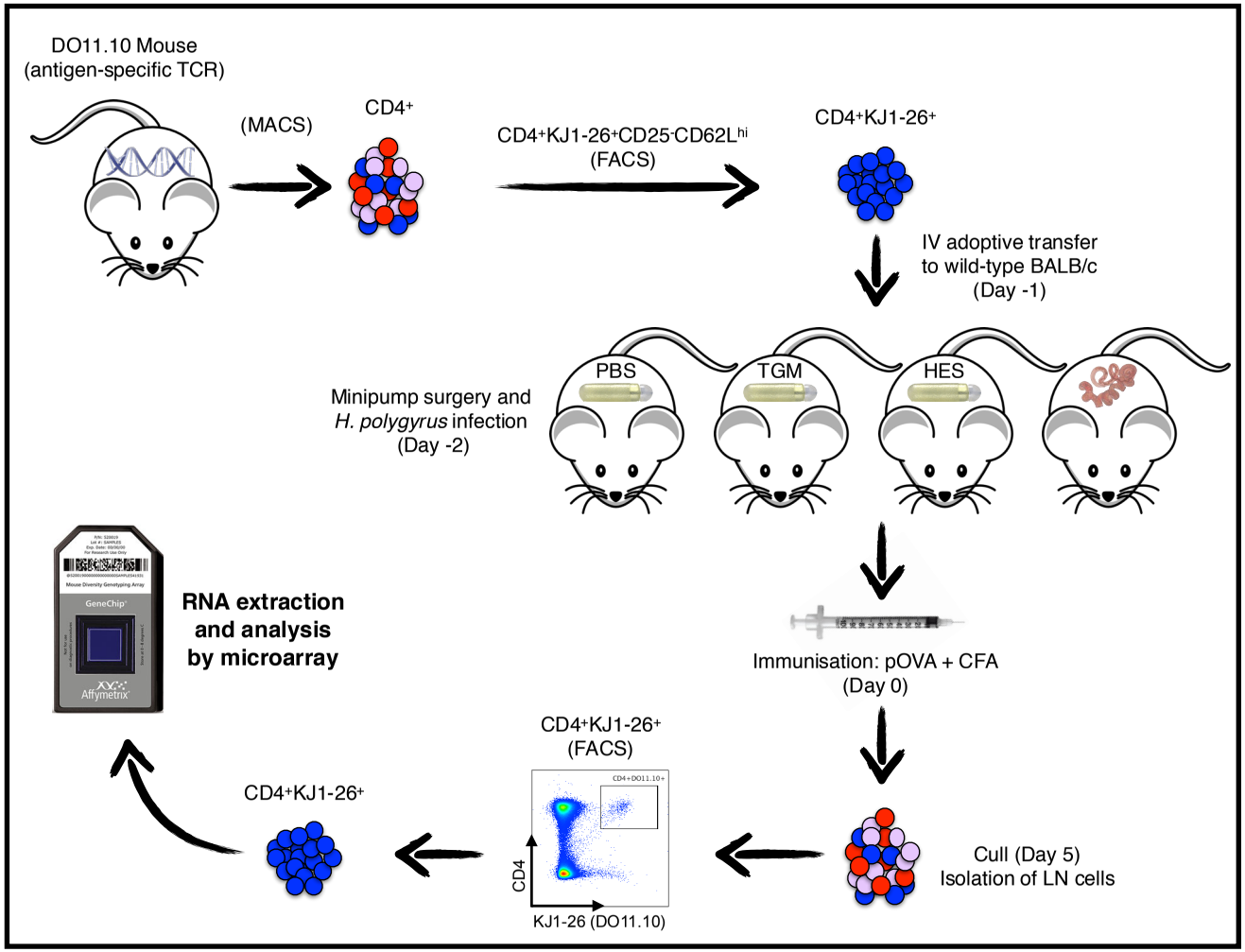


Figure 4-12 Transcriptome microarray analysis of *in vivo* stimulated antigen-specific CD4$^+$ T cells
Day -2: Four experimental groups - treatment with intraperitoneal PBS-eluting minipump (control), intraperitoneal HES-eluting minipump (2.6 μg/day), intraperitoneal TGM-eluting minipump (79.2 ng/day) and inoculation with 200 L3 *H. polygyrus* by oral gavage. Day -1: isolation of naive CD4$^+$ DO11.10 T cells by serial MACS and FACS sorting, followed by adoptive transfer (IV) of 10^6 cells into wild-type BALB/c mice. Day 0: immunisation with 20 μg pOVA and CFA containing 50 μg heat-killed *M. tuberculosis*. Day 5: cull and preparation of single cell suspensions from inguinal lymph nodes followed by FACS sorting to isolate the transferred antigen-specific DO11.10 CD4$^+$ T cell population. Cells were sorted into RNAlater and snap frozen on dry ice for subsequent RNA extraction, labeling and analysis (Affymetrix GeneChip(r) Mouse Transcriptome Pico Assay 1.0 - performed by Dr Bob Holt, Hologic Ltd).

4.3 Discussion

Infection with *H. polygyrus* powerfully supresses EAE in wild-type C57BL/6 mice and, unlike a number of other helminths that have been investigated for this ability[219-221,223], no period of prophylactic treatment is necessary to achieve protection (i.e. infection on the day of immunisation with pMOG is sufficient to considerably reduce the incidence of symptomatic disease, Figure 4-1). *Ex-vivo* recall assays demonstrated that *H. polygyrus* infection in EAE induces a significant pMOG-specific shift from Th1 and Th17 responses towards a Th2 profile. Further assessment of the ability of *H. polygyrus* infection to ameliorate EAE with parallel wild-type C57BL/6 and IL-4Rα$^{-/-}$ experimental groups revealed that a large degree of the protection afforded by *H. polygyrus* is indeed Th2-dependent. Treatment with HES was much less efficacious in suppressing EAE in wild-type C57BL/6 mice than infection with *H. polygyrus* and the degree of protection afforded to HES-treated wild-type animals closely resembled that of IL-4Rα$^{-/-}$ mice treated with *H. polygyrus* infection.

It is well established that *H. polygyrus* secretes a diverse array of immunomodulatory agents[288] and any treatment with HES or therapeutic infection is therefore likely to engage multiple regulatory mechanisms in parallel. Accurate identification of the pathophysiological processes that are unique to *H. polygyrus* infection over and above administration of HES alone is not straightforward. First, the 'secretome' of stage 4 larvae (L4) is compositionally distinct from that of adult *H. polygyrus* worms[360]. Whether any of the additionally secreted, or omitted, components of L4 secreted products are functionally significant is currently unknown. Secondly, delivery of HES by continuous infusion via an osmotic minipump provides the closest possible surrogate of HES secretion from *H. polygyrus* infection. However, in addition to a difference in the precise site of administration, the composition of HES secreted by adult *H. polygyrus* worms *in vivo* may be different to that secreted *in vitro* and numerous mediators may be lost during the HES production process or degraded during prolonged storage within the minipump prior to elution. Thirdly,

infection with *H. polygyrus* has been shown to markedly alter the gut microbiota[361], but whether this effect is due to antimicrobial products within HES, or in response to epithelial disruption by *H. polygyrus* and the reaction to it, is currently unknown. Finally, the physical trauma caused by larval migration into the duodenal submucosa and their subsequent eruption back into the intestinal lumen undoubtedly stimulate a further immune response[68], but attempts to study this in isolation by irradiating larvae (so that they can still migrate to the submucosa, but not mature into adulthood) have been frustrated by the continued ability of larvae to still secrete soluble products, even after considerable radiation exposure[362].

Data from Chapter 3 revealed that treatment with *H. polygyrus* infection induced an increase in serum IL-5 concentration (Figure 3-5) and marked Th2 deviation upon antigen restimulation (Figure 3-12), in contrast to treatment with an infusion of HES over the same period that did not. With the caveats described above notwithstanding, these observations suggest that the physical trauma caused by infection with *H. polygyrus* larvae induces a strong Th2 response, whilst exposure to HES alone does not. The striking similarity of the clinical course of EAE in wild-type mice treated with HES (Figure 4-6, A) to that of IL-4Rα$^{-/-}$ mice treated with *H. polygyrus* infection (Figure 4-3, B) also supports this hypothesis: IL-4Rα$^{-/-}$ animals treated with *H. polygyrus* are subject to the immunomodulatory effects of HES that is secreted, but not the strong Th2 response normally generated by the physical presence of the worms. Further, third stage *H. polygyrus* larvae migrate to the duodenal submucosa within 24 hours of infection[357] and, if this is the origin of a strong Th2 response, then the relatively unique ability of *H. polygyrus* to suppress established EAE within 48 hours of infection[218] could be accounted for.

PD-1 plays a critical role in regulating EAE[363] and increased expression of PD-1 by Foxp3^{-} effector CD4^{+} T cells was induced by HES in wild-type C57BL/6 mice (Figure 4-7, A) and by *H. polygyrus* infection in IL-4Rα$^{-/-}$ mice (Figure 4-5, A). This suggests that PD-1 is an immunoregulatory mechanism induced by HES in a Th2-independent fashion and may therefore account for the small, but significant, degree of protection that is afforded to IL-4Rα$^{-/-}$ mice by *H. polygyrus* and wild-type animals by HES.

Expression of the IL-33 receptor complex, ST2 (IL-1 receptor-like 1) was increased by both HES and *H. polygyrus* infection in wild type C57BL/6 mice, specifically in the $Foxp3^-$ effector $CD4^+$ T cell population. ST2 expression can facilitate Th2 polarisation and has been shown to play an important role in adaptive immunity to intestinal nematodes[364]. The fact that *H. polygyrus*-induced expression of ST2 was completely ablated in IL-4R$\alpha^{-/-}$ mice (Figure 4-4, D+F), but still inducible by HES in wild-type animals (Figure 4-7, D+F) indicates that HES does induce Th2-dependent mechanisms albeit at a comparatively low level to that of live infection.

The observation of a very small range and variance of adult *H. polygyrus* worm burdens in IL-4R$\alpha^{-/-}$ mice contrasting the wide range and variance of worm burden in wild-type C57BL/6 mice (Figure 4-3, I) is a consistent finding across multiple experiments. This suggests that the variance in worm burden in wild-type animals is not due to inconsistency in the inoculum of L3 larvae, but as a result of the immune response of the host. However, the worm burdens of individual mice have not yet yielded any correlation with functional outcome. For example, wild-type animals with the highest worm burden may be exposed to a greater concentration of HES (assuming each adult worm produces approximately equal amounts) and therefore experience the greatest degree of protection from EAE; the high worm burden may be a manifestation of successful modulation of the host immune response. Conversely, animals with the lowest worm burdens may have established the strongest Th2 response and therefore achieve a greater degree of protection from EAE as a result of cross regulation. Further study with a greater number of subjects is required.

Finally, *H. polygyrus* and HES are known to powerfully induce $Foxp3^+$ Treg[233] and this is another possible mechanism of protection from EAE that needs further investigation with assessment of T cell populations in the periphery and central nervous system at the peak of disease activity.

5 The TGF-β mimic - TGM

5.1 Introduction

The known ability of *H. polygyrus* and HES to promote Foxp3$^+$ Treg differentiation[233] without demonstrable adverse immunological sequelae presents the possibility of hitherto unidentified immunomodulators that could be used as novel therapeutic agents. As previously discussed, clinical trials of *ex-vivo* expansion and reinfusion of autologous human Treg are currently underway but face a number of challenges, including accurate identification of human Treg by cell surface markers, the possibility of infused Treg reverting to an alloantigen-specific effector T cell phenotype and the requirement for considerable logistical arrangements that would likely limit translation of this approach into routine clinical care[365]. The prospect of a pharmacological therapy to promote Treg differentiation *in vivo* therefore offers several potential advantages.

Although TGF-β is fundamental to the induction of peripheral Foxp3$^+$ Treg differentiation[317], deleterious consequences of exogenous administration including interstitial fibrosis and expansion of Th17 cells have tempered optimism for therapeutic exploitation. In addition, the regulation of TGF-β signalling is inherently complex and dependent on numerous interrelating factors, such that the pharmacokinetics of exogenous TGF-β are difficult to predict. *H. polygyrus* appears to have successfully negotiated this path, modulating the immune response of its host with TGF-β activity in HES that promotes its own ongoing survival[233], but is simultaneously not overtly detrimental to the long term wellbeing of its host.

Following the recent identification and recombinant synthesis of a TGF-β mimic within HES (TGM), this chapter seeks to identify the mechanisms by which TGM acts and to explore its immunomodulatory capabilities and potential adverse effects in comparison to exogenous mammalian TGF-β.

5.2 Results

5.2.1 TGM and HES stimulate enhanced TGF-β signalling as detected by MFB-F11 TGF-β Bioassay

The MFB-F11 cell line was developed by Tesseur *et al* by transfecting *TGFb1*$^{-/-}$ fibroblasts with a TGF-β-responsive reporter plasmid containing a secreted embryonic alkaline phosphatase reporter gene (SBE-SEAP)[351], such that TGF-β signalling is proportional to the amount of alkaline phosphatase secreted and can be measured using a p-nitrophenyl phosphate substrate and absorbance at 405 nm (as described in section 2.10; Figure 5.1, A)

Comparison of the relative MFB-F11 signal generated by half-log dilution gradients of TGF-β, TGM and HES reveals that all three induced alkaline phosphatase in a dose-dependent manner (Figure 5.1, B). The activity of TGM and TGF-β reached relative plateaus at higher concentrations, with TGM consistently inducing a significantly higher MFB-F11 signal than TGF-β, e.g. at 100 ng/ml, TGM = 2.46 ± 0.16 and TGF-β = 1.48 ± 0.02, $p = 0.02$. The MFB-F11 signal stimulated by HES also exceeded the relative plateau of the TGF-β-induced signal, but required several log-fold higher concentrations to achieve the same signal as TGM.

Following subsequent confirmation by gel filtration of TGM acting as a dimer, the MFB-F11 activity induced by TGM and TGF-β was reanalysed and presented against molar concentration for more accurate comparison (Figure 5.1, C; calculation described in section 5.2.5). As the components and relative composition of HES is unknown, similar comparison with HES is not possible.

5.2.2 TGM Induces Greater Phosphorylation of Smad2 than TGF-β

The enhanced MFB-F11 signal generated by TGM compared to TGF-β could be reflective of enhanced intracellular signalling arising from the TGF-β receptor complex, or an anomalous finding that is specific to the *TGFb1*$^{-/-}$ cells on which the MFB-F11 bioassay is based. In order to test this, splenocytes from wild type C57BL/6 mice were suspended in DMEM-2.5 and incubated for 18 hours at 37°C with 20 ng/ml TGF-β, 20 ng/ml TGM or DMEM-2.5 only (the concentration of 20 ng/ml was chosen as a level at which both TGM and TGF-β reach a saturation plateau of MFB-F11 signal). The cells were then harvested and relative amounts of Smad2 and phospho-Smad2 were assessed by western blotting, as described in section 2.17.

At 20 ng/ml TGF-β, the MFB-F11 signal had reached a relative plateau of maximal activity (Figure 5-1). Western blotting showed that 20 ng/ml TGF-β induces phosphorylation of Smad2, but that the level of Smad2 phosphorylation is higher with 20 ng/ml TGM (Figure 5-2, A-C). To address the possibility of loading error, the blot for phospho-Smad2 was stripped and reprobed to assess the total Smad2 protein present (Figure 5-2, D); normalisation of phospho-Smad2 to total Smad2 shows an identical trend (Figure 5-2, E).

5.2.3 TGM initiates signalling via the Type I TGF-β receptor, but is not neutralised by pan-vertebrate anti-TGF-β monoclonal antibody

To assess the mechanism of action of TGM, MFB-F11 cells were first co-cultured with TGM or TGF-β and 100 μg/ml pan-vertebrate anti-TGF-β antibody (clone 1D11) or MOPC-21 murine IgG control. Anti-TGF-β antibody considerably inhibited the MFB-F11 signal generated from TGF-β, but had no impact on TGM (Figure 5-3, A and B). Next, a potent inhibitor of the Type I TGF-β receptor kinase,

SB431542, was added to culture (5 μM final concentration) and found to completely ablate the MFB-F11 signal from both TGF-β and TGM (Figure 5-3, C and D).

Seven mammalian variants of the Type I TGF-β receptor have been identified and are known as activin receptor-like kinases (ALK) 1-7. Of these, SB431542 inhibits ALK4, ALK5 and ALK7[366]. TGF-β and the majority of other TGF-β superfamily ligands signal through ALK5.

This experiment demonstrated that TGM ligates a TGF-β receptor complex that incorporates ALK4, ALK5 or ALK7, but is sufficiently structurally distinct from TGF-β as to avoid the binding of an antibody that recognises bovine, murine and all three human forms of TGF-β.

5.2.4 TGM inhibits proliferation of naive $CD4^+$ responder T cells

Having established that TGM ligates the TGF-β receptor complex, the functional significance of this was first explored by assessing if TGM could inhibit *in vitro* $CD4^+$ T cell proliferation in a similar fashion to TGF-β[367]. A naïve $CD4^+$ responder T cell population was isolated from Foxp3-GFP transgenic mice by CD4 MACS positive selection, followed by flow cytometric sorting, gating on $CD4^+CD25^-GFP^-CD62L^{hi}$. Proliferation was assessed by thymidine incorporation after 72 hours (Figure 5-4, A-B; method – section 2.9).

TGM was found to inhibit CD3-mediated proliferation of naïve murine $CD4^+$ T cells in a concentration-dependent fashion, with a maximal observed suppression of 88.2% at a concentration of 50 ng/ml (Figure 5-4, C).

5.2.5 TGM induces *de novo* Foxp3 expression in naive CD4$^+$ T cells and induces greater Foxp3 expression than TGF-β at high concentrations

H. polygyrus and HES have previously been shown to induce Foxp3$^+$ Treg *in vitro* and *in vivo*[233]. After demonstrating that TGM acts in a TGF-β-like fashion and stimulates enhanced downstream signalling (as evidenced by MFB-F11 bioassay signal and phosphorylation of Smad2), the next set of experiments was designed to establish a) if TGM could induce Foxp3$^+$ Treg differentiation *in vitro* and b) if the previously observed evidence of enhanced intracellular signalling would be reflected in the level of Foxp3 expression.

CD4$^+$CD25$^-$GFP$^-$CD62L^{hi} cells were isolated by MACS and FACS sorting as before (section 5.2.4) and cultured for 96 hours in Treg polarising conditions (section 2.11) with half-log dilution gradients of TGF-β or TGM. TGM was found to effectively induce Foxp3$^+$ Treg differentiation: 390 nM (38.1 ng/ml) induced Treg conversion in 90.65% (± 3.55%) of all CD4$^+$ cells, compared to 79.65% (± 2.55%) induced by 390 nM (10 ng/ml) TGF-β (Figure 5-5, A-C). Further, the mean fluorescence intensity of Foxp3 expression induced by high concentrations of TGM was found to be greater than that of the equivalent concentration of TGF-β (Figure 5-5, D-E). This result correlates with previous experiments showing evidence of 'enhanced' intracellular TGF-β signalling achieved by TGM compared to TGF-β itself.

5.2.6 TGM inhibits pro-inflammatory cytokine production from CD3/CD28-stimulated CD4$^+$ T cells

In addition to analysis of Foxp3 expression, culture supernatants from the Treg induction assays described previously (section 5.2.5) were tested for the presence of IFNγ, IL-13, IL-17 and TNFα by cytokine bead array (section 2.16). All four cytokines were powerfully inhibited by TGM and TGF-β in a concentration-dependent fashion (Figure 5-6), indicating another mechanism through which TGM can suppress inflammation.

5.2.7 TGM-induced Foxp3 expression is dependent on the Type I TGF-β receptor

To further characterise the mechanism of TGM-mediated Foxp3 expression, Treg induction assays with TGM and TGF-β (section 2.11) were repeated with the addition of SB431542 inhibitor, pan-vertebrate anti-TGF-β antibody, or appropriate controls. TGF-β-mediated Foxp3 induction was considerably inhibited by both SB431542 and anti-TGF-β antibody (Figure 5-7, A and B); in contrast, while TGM-mediated Foxp3 induction was inhibited by SB431542, anti-TGF-β antibody had no effect (Figure 5-7, C and D). This finding confirms that, despite the apparent greater efficacy of TGM in inducing Foxp3 compared to TGF-β, TGM-mediated Foxp3 induction is like mammalian TGF-β, wholly dependent on the Type I TGF-β receptor (ALK4, 5 or 7).

5.2.8 TGM-generated Foxp3$^+$ Treg are functionally suppressive *in vitro*

The level of Foxp3 expression by Treg has previously been shown to directly correlate with their suppressive capacity *in vitro* and *in vivo*[333]. Having found that high concentrations of TGM induce greater Foxp3 expression in Treg than the equivalent concentration of TGF-β, it was hypothesized that TGM-induced Treg are functionally more suppressive than TGF-β-induced Treg.

Treg were generated under identical conditions to the assay performed in Figure 5-5 with 'maximal' concentrations of TGF-β and TGM (10 ng/ml and 38.2 ng/ml, respectively). Treg from respective cultures were then isolated by FACS with a consistent gating strategy of $CD4^+CD25^+GFP^+$ cells with a high degree of purity. As a control population, nTreg were isolated from freshly prepared Foxp3-GFP transgenic mouse splenocytes using the same gating strategy.

TGF-β-generated Treg, TGM-generated Treg and nTreg were placed in parallel Treg suppression assays with soluble CD3, irradiated APC and $CD4^+CD25^-GFP^-CD62L^{hi}$

responder cells (as described in section 2.12). Assessment of responder cell proliferation by thymidine incorporation demonstrated that TGM-generated Treg are functionally suppressive *in vitro* but, under these conditions, suppressive capacity was equivalent to TGF-β-generated Treg (figure 5-8, A-G).

5.2.9 Polyclonal anti-TGM antibody neutralises TGM, but not TGF-β or HES

To further delineate the relative contribution of TGM to the immunosuppressive capabilities of HES, a polyclonal rat IgG antibody was raised to the recombinant TGM protein (Figure 5-9, A - performed by Yvonne Harcus). Anti-TGM antibody (purified IgG fraction) was then added to MFB-F11 bioassay cultures with serial dilutions of TGF-β, TGM and HES. Anti-TGM antibody neutralised the MFB-F11 signal from TGM in a concentration-dependent fashion (Figure 5-9A), but had little effect on TGF-β (Figure 5-9 B) and, interestingly, also had little impact on HES (Figure 5-9, C).

Although at first sight, this result would argue (a) that there is no shared epitope between TGM and TGF-β, and (b) that HES contains additional 'TGF-β mimics' to TGM, an important caveat must be noted. The recombinant TGM protein is expressed from the pSec-Tag vector which includes both the c-Myc-tag and 6-His-tag, which may elicit most or all of the polyclonal antibody response. Generation of monoclonal antibodies to the TGM itself will be required to resolve this issue.

5.2.10 TGM and TGF-β signal exclusively via the Type II TGF-β receptor; HES does not

The possibility of additional active 'TGF-β mimics' was further explored with the use of an additional receptor inhibitor – 'Inducer of Type II TGF-β Receptor Degradation-1' (ITD-1). As previously discussed, TGF-β superfamily ligands signal through a TGF-β receptor complex that is formed as a heterodimer containing one

type I TGF-β receptor and one type II TGF-β receptor. Five forms of the type II TGF-β receptor are known: TGF-β receptor II (TβRII) activin receptor II, activin receptor IIb, bone morphogenic protein receptor II (BMPRII) and anti-Müllerian hormone receptor II (AMHRII)[366]. Of these subtypes, ITD-1 specifically induces proteolytic degradation of TβRII[368] (Figure 5-10, A).

ITD-1 or an equivalent concentration of DMSO control was added to MFB-F11 bioassay cultures with serial dilutions of TGF-β, TGM and HES. Interestingly, ITD-1 completely ablated the MFB-F11 signal generated by TGF-β (figure 5-10, B) and TGM (figure 5-10, C), but not that of HES (figure 5-10, D). This result suggests that HES does indeed contain one or more active additional TGF-β mimics that signals via one of the four type II TGF-β receptor subtypes other than TβRII. Notably, the MFB-F11 bioassay does not respond to activin[351] and therefore additional ligands within HES are likely to act via a TGF-β receptor complex that incorporates BMPRII or AMHRII.

5.2.11 TGM inhibits proliferation of human $CD4^+$ T cells

As TGM is derived from a murine parasite (*H. polygyrus*) and shares no sequence homology with mammalian TGF-β (or any other recognized protein), the degree of species-specificity in its mechanism of action was unknown.

To test this, human $CD4^+$ T cells were isolated from peripheral blood by means of a Ficoll gradient followed by MACS positive selection (sections 2.7.2-3). Cells were then labelled with CellTrace® violet and placed in culture at 37°C for 96 hours with a 1:1 ratio of CD3/CD28 Dynabeads® (section 2.9) and variable concentrations of TGM or TGF-β. At the end of the culture period, cells were acquired on a BD LSR Fortessa for assessment of proliferation by generational tracing. As shown in Figure 5-11, TGM was found to inhibit proliferation in a concentration-dependent fashion to a similar degree as TGF-β, although in neither case was the effect as profound as might have been expected. Addition of SB431542 ablated the suppression of

proliferation, confirming that this effect of TGM (and TGF-β) is mediated via the type I TGF-β receptor (Figure 5-11, A).

5.2.12 TGM induces Foxp3 expression in human $CD4^+$ T cells

Having found that TGM and TGFβ inhibited proliferation of human $CD4^+$ T cells to a similar extent, cells from section 5.2.11 were also stained for CD25 (PE) and Foxp3 (AF647) expression to assess the ability of TGM to promote human Treg differentiation. As shown in Figure 5-12(A) the proportion of $CD4^+CD25^+Foxp3^+$ Treg increased with TGM in a concentration-dependent fashion, to a maximum of 84% (± 2.5%). The proportion of Treg of all $CD4^+$ cells was similar for TGM and TGF-β at equivalent molar concentrations; however, at higher concentrations, the mean fluorescence intensity of Foxp3 expression was significantly greater in Treg exposed to TGM compared to TGF-β (e.g. at 12nM, MFI of 1369.5 (± 70.5) vs. 1082 (± 27), $p < 0.001$; Figure 5-12, B-C).

5.2.13 TGM inhibits proliferation of human $CD8^+$ T cells

In view of the importance of CD8+ T cells in many immune-mediated pathologies including allograft rejection, the impact of TGM on the proliferation of human $CD8^+$ T cells was also assessed. $CD8^+$ cells were isolated by MACS positive selection and labelled with CellTrace® violet as before (section 5.2.11). Cells were placed in culture at 37°C for 96 hours with variable concentrations of CD3/CD28 Dynabeads® with or without TGM or TGF-β; at the time of this experiment the dimeric structure, and therefore molarity, of TGM was not known and both proteins were tested at 10 ng/ml. Notably, TGM effectively suppressed $CD8^+$ T cell proliferation at all concentrations of stimulating beads (Figure 5-13).

5.2.14 TGM induces myofibroblast differentiation less potently than TGF-β

Despite sharing no sequence homology with TGF-β, work so far presented establishes that TGM ligates the TGF-β receptor complex and shows TGF-β-like activity on both mouse and human cells. As a result, the pro-fibrotic effect that is seen with TGF-β[369] was predicted as an adverse effect that might limit the therapeutic potential of TGM.

To address this, a collaboration was established with Dr Hannah Woodcock (Prof Rachel Chambers' laboratory, University College London) to assess the ability of TGM to induce myofibroblast differentiation in comparison to TGF-β. Macromolecular crowding assays with human lung fibroblasts were performed with equivalent molar concentrations of TGM or TGF-β (section 2.14) to assess for expression of alpha-smooth muscle actin and extracellular deposition of type I collagen.

This approach revealed that TGM does induce myofibroblast differentiation in human lung fibroblasts; however, the dose-response relationship for both alpha-smooth muscle actin (Figure 5-14, A-C) and type I collagen (Figure 5-15, A-C) shows that TGM is less potent in this respect than TGF-β. Most notably, this relationship is the opposite of that seen with Foxp3 induction in both mouse and human cells.

5.2.15 TGM prolongs survival of fully-allogeneic skin grafts

To assess the functional impact of TGM *in vivo*, full-thickness allogeneic skin grafts were performed from BALB/c tail skin onto the left flank of C57BL/6 recipient mice, that either received no treatment, or an intraperitoneal minipump eluting 79.2 ng/day of TGM (implanted immediately prior to skin grafting). Dressings were removed 7 days after transplantation and grafts were then monitored on a daily basis

until complete rejection, which was defined as more than 90% necrosis by surface area, or complete dehiscence.

Treatment with TGM significantly prolonged allograft survival, with a median survival time of 13 days compared to 9 days for untreated allograft recipients ($p = 0.0136$ as assessed by the Mantel-Cox test) and a hazard ratio of 0.342 (95% CI: 0.034 – 0.463) (Figure 5-16).

Control syngeneic (C57BL/6 to C57BL/6) skin grafts showed no signs at all of rejection at the termination of the experiment on day 21 post transplantation ($n = 3$).

5.2.16 TGM induces Foxp3 expression by $CD4^+$ T cells *in vivo*

To investigate the influence of TGM on the alloimmune response *in vivo*, cell populations from the spleen and allograft draining lymph node of skin graft recipient animals were analysed by flow cytometry. Two experiments were performed under identical conditions to allow for cellular analysis at 7 and 21 days after transplantation.

Full-thickness BALB/c skin grafts were transplanted on to C57BL/6 mice in two experimental groups: no treatment, or implantation of an intraperitoneal osmotic minipump 79.2 ng/day of TGM immediately prior to transplantation. Syngeneic C57BL/6 to C57BL/6 skin grafts were also performed in each experiment to provide control cell populations.

The mean percentage of $CD4^+$ T cells expressing Foxp3 within the spleen and allograft draining lymph node was found to be greater in allograft recipients receiving TGM compared to untreated animals. A summary of the proportions of $CD4^+Foxp3^+$ cells, together with fold change as a result of TGM treatment and associated p values (as assessed by two-tailed, unpaired t tests) is presented in Table 5-1.

Cell population	Allograft only %$CD4^+Foxp3^+$ of all $CD4^+$ cells	+TGM %$CD4^+Foxp3^+$ (fold change compared to no treatment; p value)
Day 7 LN	14.63	15.75 (1.08; p = 0.131)
Day 7 Spleen	21.82	24.67 (1.13; **p = 0.0135**)
Day 21 LN	14.23	16.23 (1.14; **p = 0.0042**)
Day 21 Spleen	18.7	21.08 (1.13; **p = 0.0025**)

Table 5-1 Foxp3 expression by $CD4^+$ T cells of allograft recipients (comparison following treatment with TGM)

5.2.17 TGM suppresses expression of Tbet by $CD4^+$ T cells

$CD4^+$ T cell populations from the spleen and allograft draining lymph node of skin graft recipients described in Section 5.2.16 (BALB/c skin grafts on to C57BL/6 mice with either no treatment or 79.2 ng/day of TGM, in addition to C57BL/6 to C57BL/6 syngeneic control grafts) were assessed for expression of Tbet by flow cytometry.

Treatment with TGM was found to significantly suppress Tbet expression by $CD4^+$ T cells in the spleen and allograft draining lymph node of allograft recipients at both 7 and 21 days after transplantation. A summary of the proportions of $CD4^+Tbet^+$ cells, together with fold change as a result of TGM treatment and associated p values (as assessed by two-tailed, unpaired t tests) is presented in Table 5-2.

Cell population	Allograft only %$CD4^+Tbet^+$ of all $CD4^+$ cells	+TGM %$CD4^+Tbet^+$ (fold change compared to no treatment; p value)
Day 7 LN	61.75	33.23 (0.54; **p < 0.0001**)
Day 7 Spleen	19.72	11.05 (0.56; **p = 0.0001**)
Day 21 LN	59.62	39.3 (0.66; **p < 0.0001**)
Day 21 Spleen	52.79	39.6 (0.75; **p = 0.0018**)

Table 5-2 Tbet expression by $CD4^+$ T cells of allograft recipients (comparison following treatment with TGM)

5.2.18 TGM suppresses expression of ROR-γt by $CD4^+$ T cells

$CD4^+$ T cell populations from the spleen and allograft draining lymph node of skin graft recipients described in Section 5.2.16 (BALB/c skin grafts on to C57BL/6 mice with either no treatment or 79.2 ng/day of TGM, in addition to C57BL/6 to C57BL/6

syngeneic control grafts) were further assessed for expression of ROR-γt by flow cytometry.

Treatment with TGM was found to significantly suppress ROR-γt expression by $CD4^+$ T cells in the allograft draining lymph node of allograft recipients 7 days after transplantation, and to suppress expression by splenic $CD4^+$ T cells at both 7 and 21 days after transplantation. A summary of the proportions of $CD4^+$ROR-γt^+ cells, together with fold change as a result of TGM treatment and associated p values (as assessed by two-tailed, unpaired t tests) is presented in Table 5-3.

Cell population	Allograft only %$CD4^+$ROR-γt^+ of all $CD4^+$ cells	+TGM %$CD4^+$ROR-γt^+ (fold change compared to no treatment; p value)
Day 7 LN	6.18	3.86 (0.62; **p = 0.0001**)
Day 7 Spleen	6.24	5.44 (0.87; **p = 0.0001**)
Day 21 LN	26.85	19.11 (0.71; p = 0.326)
Day 21 Spleen	14.93	5.472 (0.37; **p = 0.0012**)

Table 5-3 ROR-γt expression by $CD4^+$ T cells of allograft recipients (comparison following treatment with TGM)

5.2.19 TGM suppresses expression of GATA3 by $CD4^+$ T cells

H. polygyrus and HES were previously shown to exert different effects on splenic $CD4^+$ T cells in respect to Th2 polarisation (Figure 3-11 and Figure 3-12). To assess the impact of TGM on Th2 polarisation, $CD4^+$ T cells from the spleen and allograft draining lymph node of skin graft recipients (BALB/c skin grafts on to C57BL/6 mice with either no treatment or 79.2 ng/day of TGM, in addition to C57BL/6 to C57BL/6 syngeneic control grafts) were assessed for GATA3 expression at 21 days

after transplantation. Flow cytometric analysis revealed that TGM significantly suppresses GATA3 expression by $CD4^+$ T cells in both the allograft draining lymph node (0.49-fold change, $p < 0.0001$) and the spleen (0.73-fold change, $p = 0.0093$).

5.2.20 TGM suppresses IFNγ and IL-4 production upon restimulation

To assess the affect of TGM on antigen-specific immune responses, splenocytes harvested from allograft recipients 21 days after transplantation were restimulated under multiple conditions in parallel for 72 hours at 37°C. Experimental conditions were the same as those described in section 5.2.19 (allografts with no treatment or treatment with 79.2 ng/day TGM by continuous infusion, in addition to syngeneic graft controls).

Restimulation with irradiated BALB/c (donor allogeneic) cells revealed a significant reduction of IFN-γ production (measured in the culture supernatant by ELISA) by splenocytes from allograft recipients treated with TGM compared to those that received no treatment (0.55-fold change, $p = 0.0018$; Figure 5-21, A). The same effect on IFN-γ production was observed following restimulation with soluble CD3 (0.55-fold change, $p < 0.0001$; Figure 5-21, A) and correlates with expression of Tbet by $CD4^+$ T cells 21 days after transplantation (Figure 5-18, D).

Measurement of IL-4 following restimulation with irradiated BALB/c (donor allogeneic) cells revealed significant suppression of IL-4 production from splenocytes of TGM-treated allograft recipients compared to untreated animals (0.13-fold change, $p = 0.0006$; Figure 5-21, B). The same effect was seen following restimulation with soluble CD3 and culture with syngeneic C57BL/6 cells (Figure 5-21, B). TGM-induced suppression of IL-4 secretion is similar to the effect of HES (Figure 3-12) and correlates with TGM-induced suppression of GATA3 expression by $CD4^+$ T cells 21 days after transplantation (Figure 5-20).

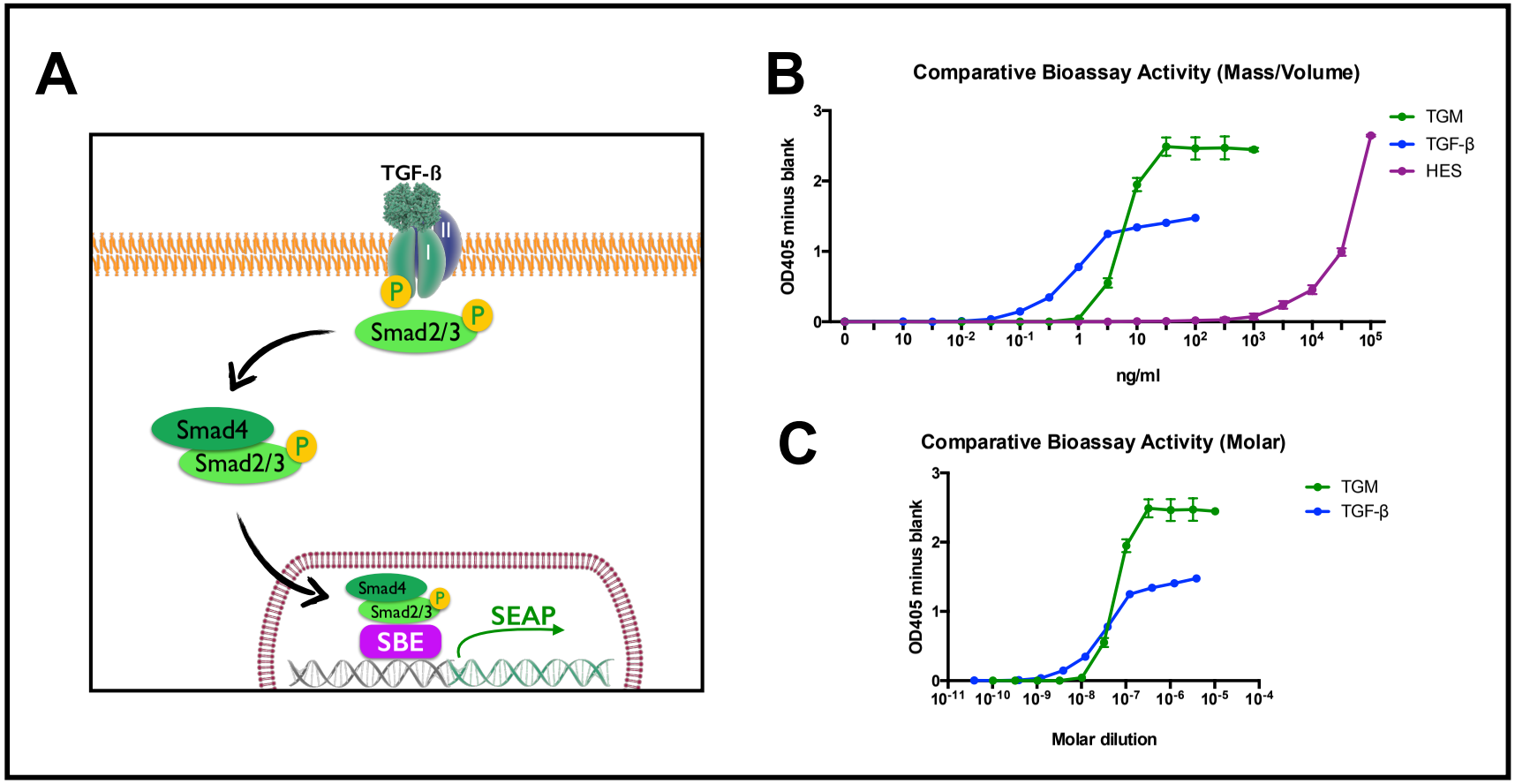


Figure 5-1 TGM and HES stimulate enhanced TGF-β signaling as detected by MFB-F11 TGF-β Bioassay
[SEAP - secreted embryonic alkaline phosphatase; SBE - Smad binding element]
(A) Schematic of TGF-β-responsive bioassay developed by Tesseur *et al* by transfecting MFB-F11 TGF-β-/- fibroblasts with a reporter plasmid consisting of a Smad binding element coupled to a secreted alkaline phosphatase reporter gene. Ligation of the type II TGF-β receptor by TGF-β leads to phosphorylation of the intracellular component of the type I TGF-β receptor and subsequently phosphorylation of the Smad2/3 complex. Phosphorylated Smad2/3 then binds Smad4 and translocates to the nucleus, leading to secretion of alkaline phosphatase, which is measured following the addition of p-nitrophenyl phosphate.
(B) MFB-F11 bioassay activity following 24 hours of culture at 37°C with TGF-β, TGM or HES. (C) MFB-F11 bioassay activity following 24 hours of culture at 37°C with TGF-β or TGM - plot as activity against molarity (the number and proportion of active mediators within HES is unknown and therefore molarity cannot be calculated).

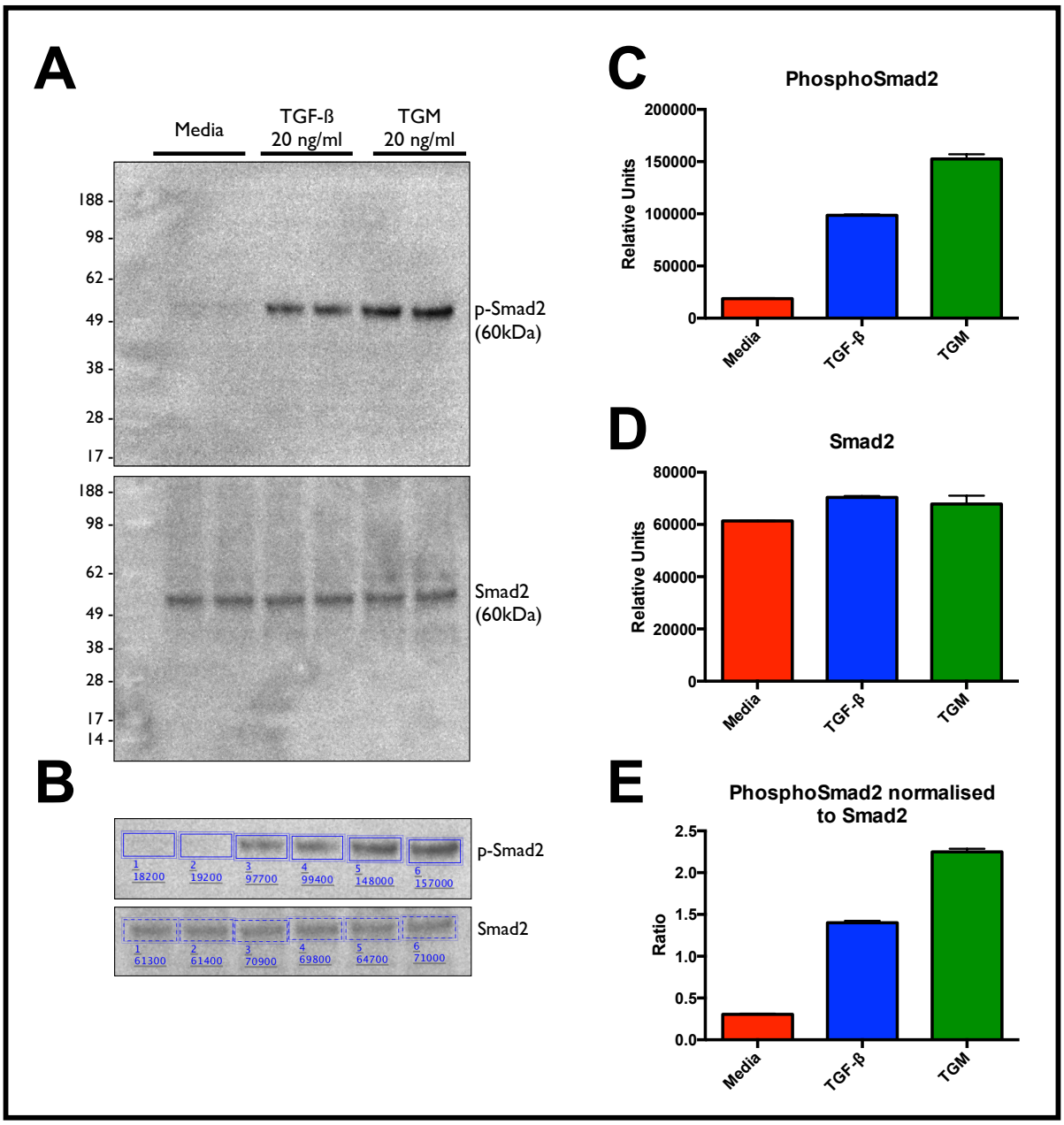


Figure 5-2 TGM Induces Greater Phosphorylation of Smad2 than TGF-β
(A) Western blots (Smad2 and phospho-Smad2): cell lysates from C57BL/6 splenocytes following culture at 37°C for 18 hours. Culture conditions in duplicate: media (DMEM-2.5), media supplemented with 20 ng/ml TGF-ß and media supplemented with 20 ng/ml TGM.
(B) Densitometry analysis of bands with Image Studio (version 5, Li-Cor).
(C) Densitometric quantification of phospho-Smad2.
(D) Densitometric quantification of Smad2.
(E) Ratio of phospho-Smad2 normalised to Smad2.
[Data shown arising from a single experiment]

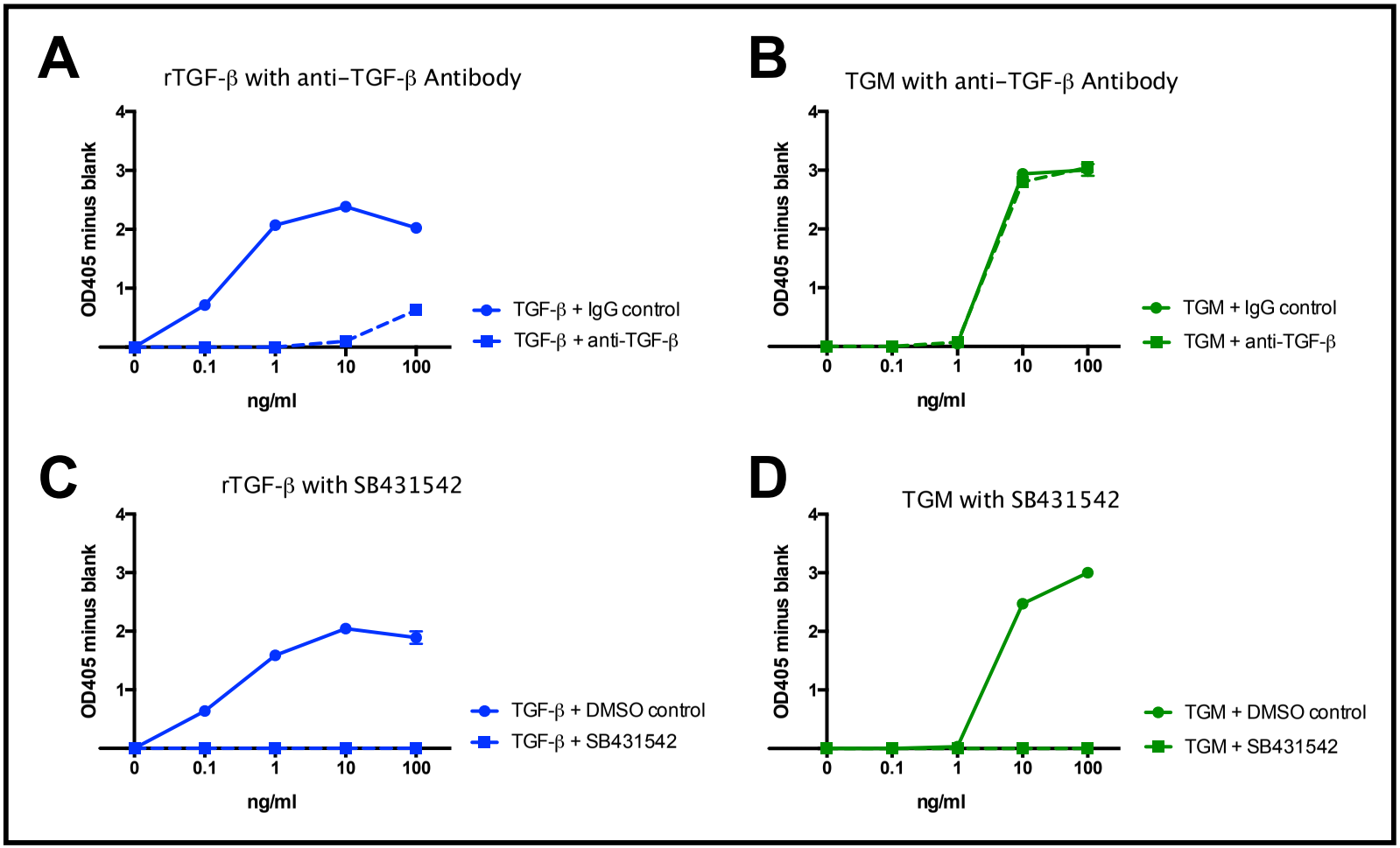


Figure 5-3 TGM initiates signaling via the Type I TGF-β receptor, but is not neutralised by pan-vertebrate anti-TGF-β monoclonal antibody

All graphs: activity shown from MFB-F11 bioassay (as described in figure 5-1) after 24 hours of culture at 37°C with:
(A) TGF-β and anti-TGF-β monoclonal antibody (interrupted line) or MOPC IgG control (solid line).
(B) TGM and anti-TGF-β monoclonal antibody (interrupted line) or MOPC IgG control (solid line).
(C) TGF-β and the type I TGF-β receptor inhibitor, SB431542 (interrupted line) or DMSO control (solid line).
(D) TGM and SB431542 (interrupted line) or DMSO control (solid line).
Data shown is representative of two independent experiments.

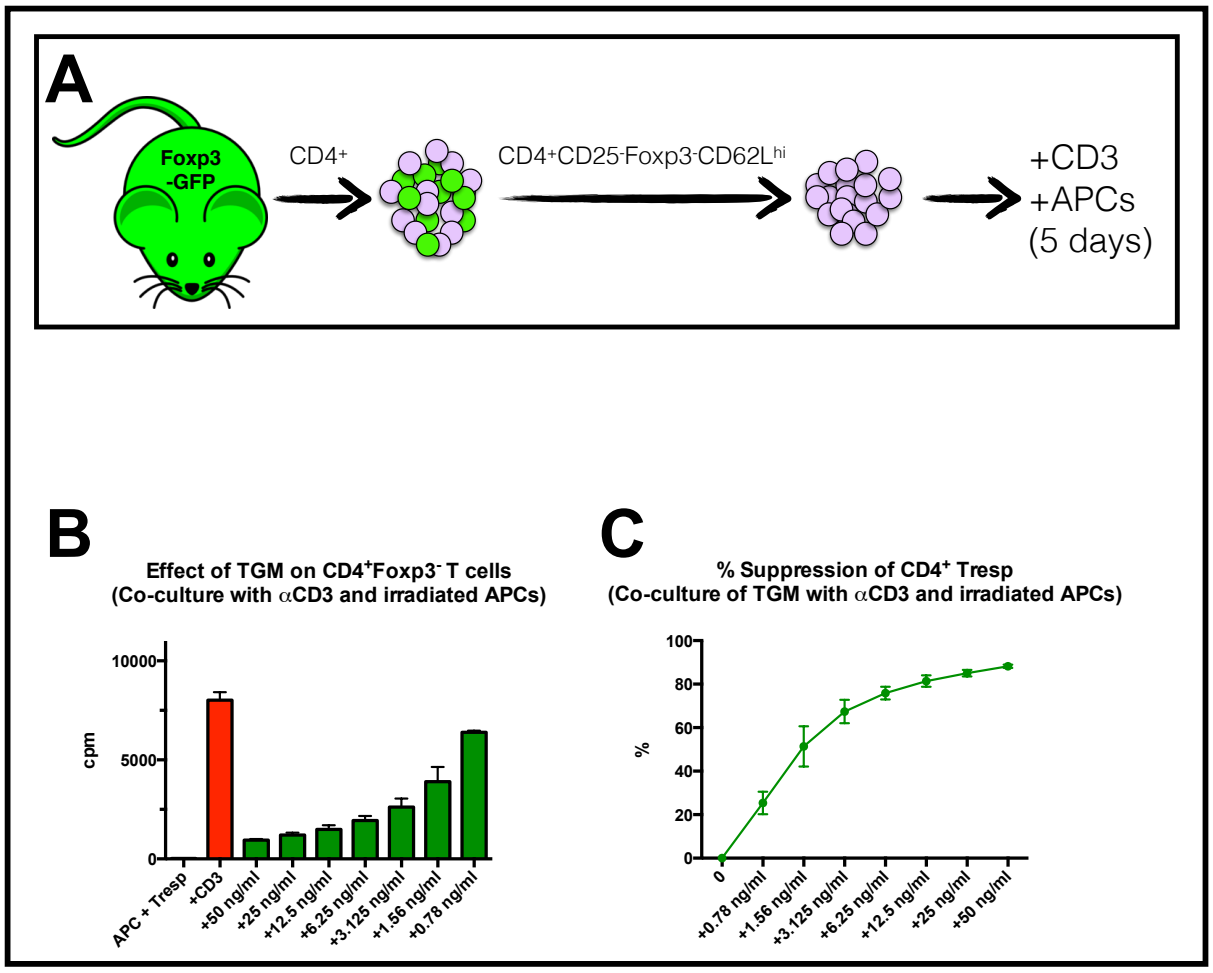


Figure 5-4 TGM inhibits proliferation of naive CD4$^+$ responder T cells
(A) Experimental design: a single cell suspension from Foxp3-GFP reporter mouse spleen and peripheral lymph nodes was enriched for CD4$^+$ cells by MACS positive selection and then sorted for CD4$^+$CD25$^-$GFP$^-$CD62L^{hi} cells by FACS to provide a naive CD4$^+$ responder cell population. The negative fraction of the MACS sort was irradiated (30 Gy) to provide a population of APCs without the capacity to proliferate. In triplicate wells of a 96 well plate round bottom plate, 10^4 responder cells were added to 10^5 APCs, 1 µg/ml CD3 and a variable concentration of TGM. Proliferation was assessed by adding thymidine for the last 18 hours of a 5 day culture at 37°C and measuring H^3 incorporation.
(B) Proliferation according to thymidine (H^3) incorporation.
(C) Percentage suppression of proliferation against concentration of TGM in culture.

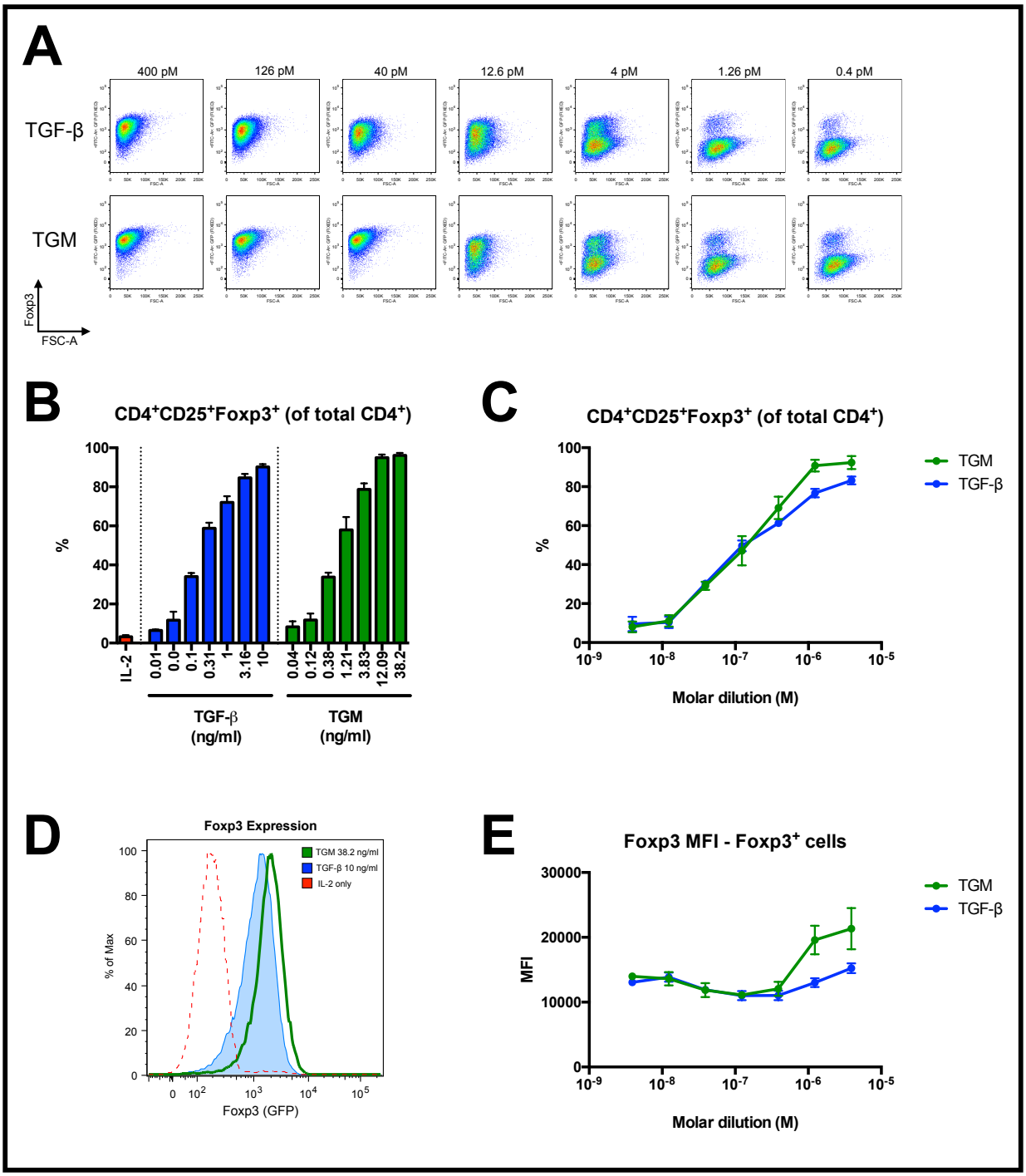


Figure 5-5 TGM induces *de novo* Foxp3 expression in naive CD4⁺ T cells and induces greater Foxp3 expression than TGF-β at high concentrations

(A) $CD4^+CD25^-GFP^-CD62L^{hi}$ naive T cells were isolated as before (figure 5-4) and stimulated with plate-bound CD3/CD28 for 4 days in culture with 100 U/ml IL-2 and variable concentrations of TGF-β or TGM. Flow cytometry plots from the end of the culture period ($CD4^+$ population shown).

(B and C) Treg induction (expressed as a percentage of total $CD4^+$ cells)

(D) Histogram overlay showing Foxp3 expression following culture conditions described above with comparison of 400 µM TGM, 400 µM TGF-β, or 100 U/ml IL-2 only.

(E) Mean fluorescence intensity of Foxp3 expression (of $Foxp3^+$ cells) against molar dilutions of TGM and TGF-β.

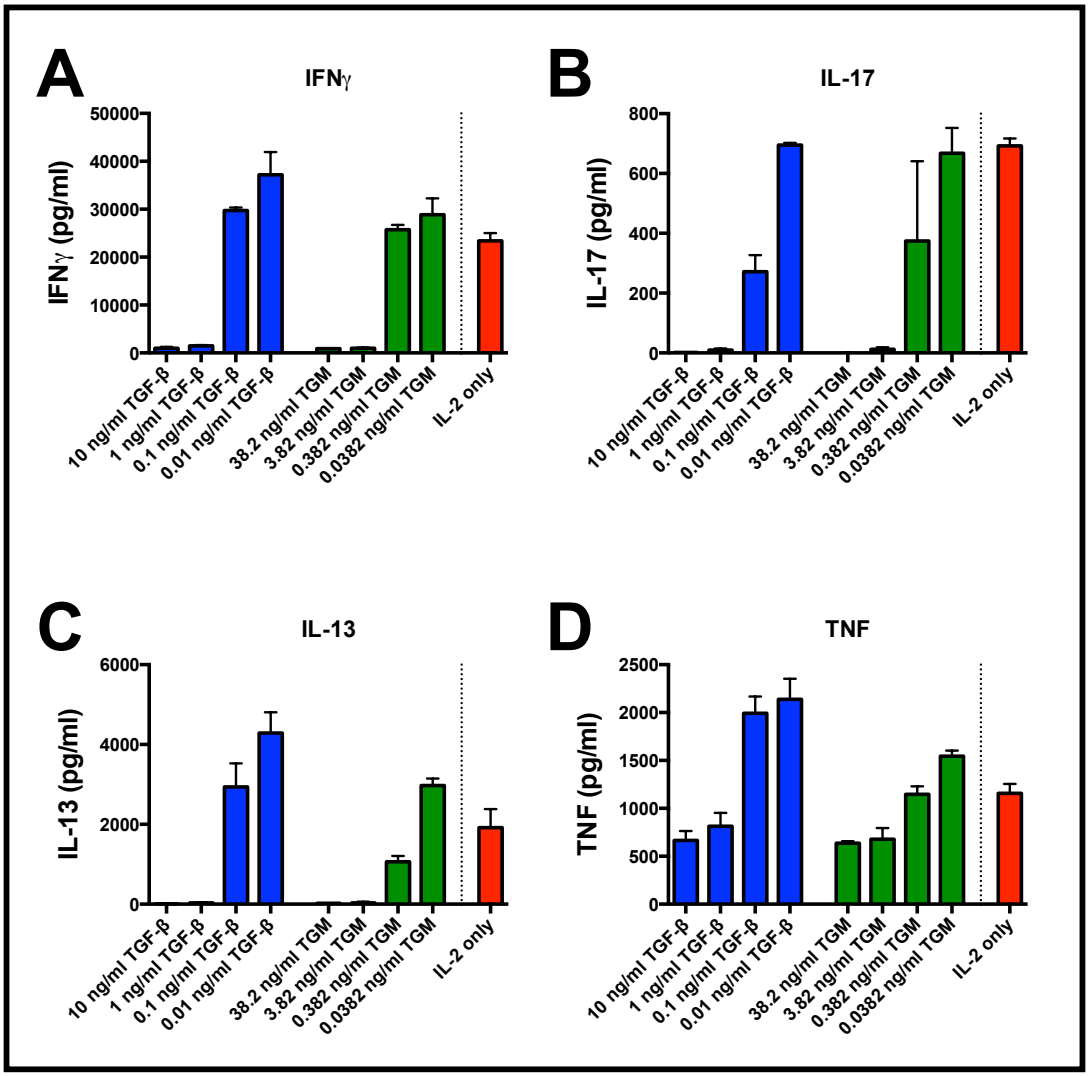


Figure 5-6 TGM inhibits pro-inflammatory cytokine production from CD3/CD28-stimulated CD4$^+$ T cells

Concentrations of (A) IFNγ, (B) IL-17, (C) IL-13 and (D) TNFα present in culture supernatants from Treg induction assays shown in figure 5-5, measured by cytokine bead array. Concentrations of TGF-β and TGM are based on molar equivalence.

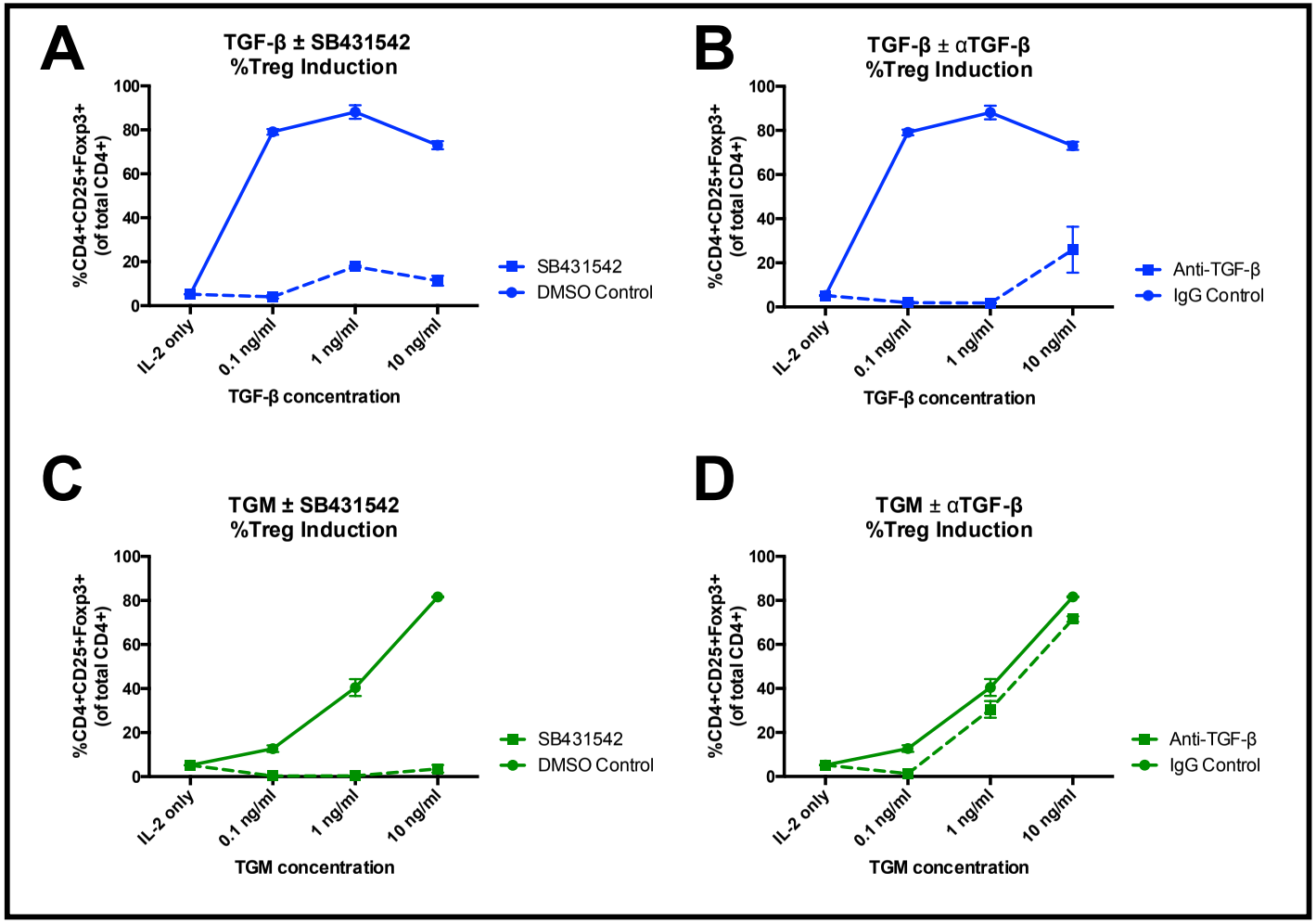


Figure 5-7 TGM-induced Foxp3 expression is dependent on the Type I TGF-β receptor

$CD4^+CD25^-GFP^-CD62L^{hi}$ naive T cells were isolated as before (figure 5-4) and stimulated with plate-bound CD3/CD28 for 4 days in culture with 100 U/ml IL-2 and variable concentrations of TGF-β or TGM + treatment. For each plot, the percentage $CD4^+CD25^+Foxp3^+$ Treg of all $CD4^+$ cell is shown.

(A) TGF-β + monoclonal antibody to TGF-β (interrupted line) or IgG control (solid line). (B) TGM + monoclonal antibody to TGF-β (interrupted line) or IgG control (solid line). (C) TGF-β + SB431542 Type I TGF-β receptor inhibitor (interrupted line) or DMSO control (solid line). (D) TGM + SB431542 (interrupted line) or DMSO control (solid line).

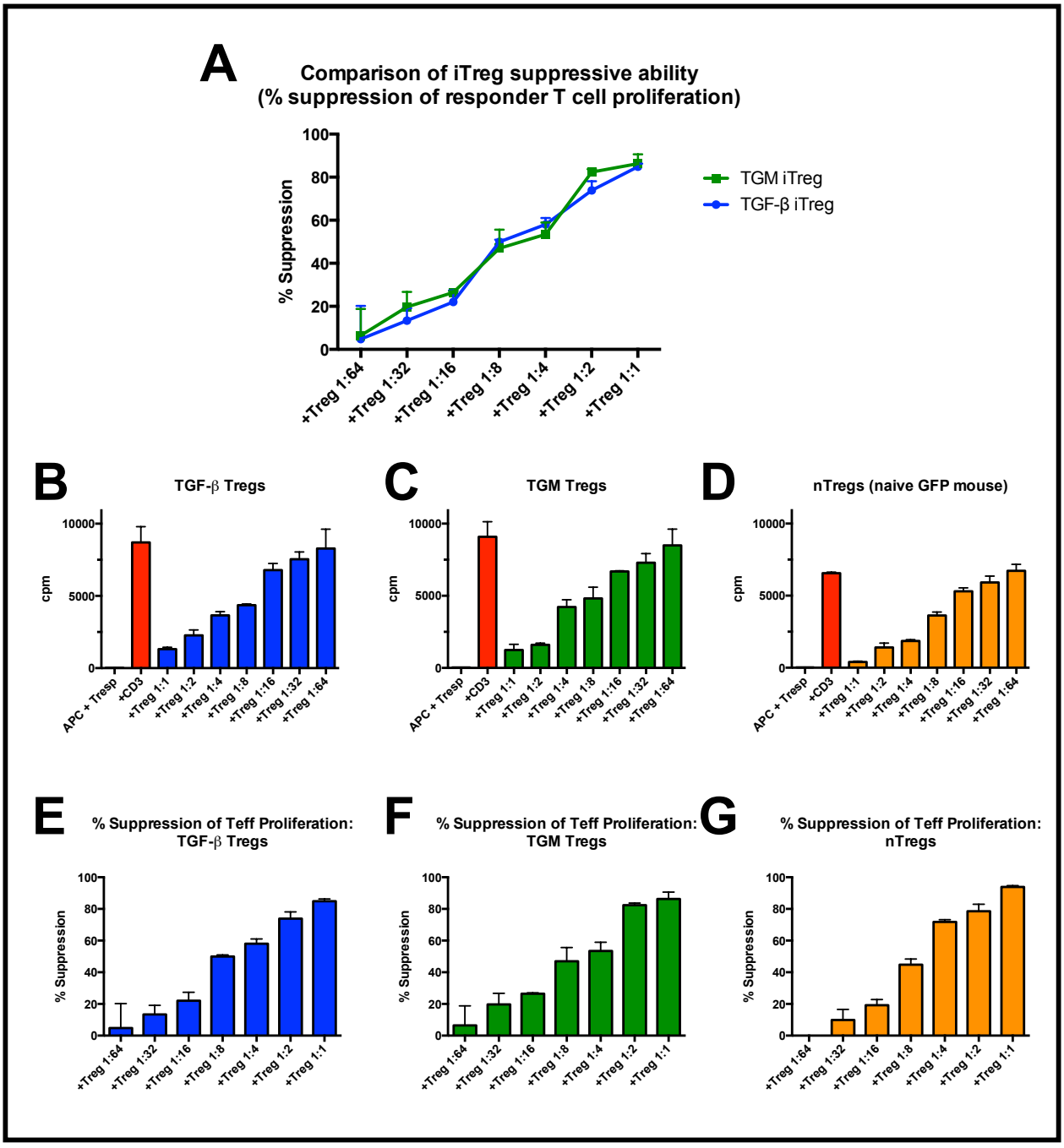


Figure 5-8 TGM-generated Foxp3$^+$ Treg are functionally suppressive *in vitro*

Foxp3$^+$ Treg were generated in vitro (as described in figure 5-4) with 38.2 ng/ml TGM or 10 ng/ml TGF-β. After four days of culture, CD4$^+$CD25$^+$GFP$^+$ cells were isolated by FACS. A single cell suspension was then freshly prepared from Foxp3-GFP mice to provide naive CD4$^+$CD25$^-$GFP$^-$ responder cells and CD4$^+$CD25$^+$GFP$^+$ tTreg as a control population. Treg were cultured with responder cells, irradiated APCs and soluble CD3 for 5 days and proliferation was assessed by thymidine incorporation (section 2.9).
(A) Comparison of percentage suppression (in relation to responder cells with APC, CD3 and no Treg) of TGM-generated iTreg and TGF-β-generated iTreg. (B-D) Proliferation as assessed by thymidine incorporation. (E-G) Percentage suppression (in relation to responder cells with APC, CD3 and no Treg).

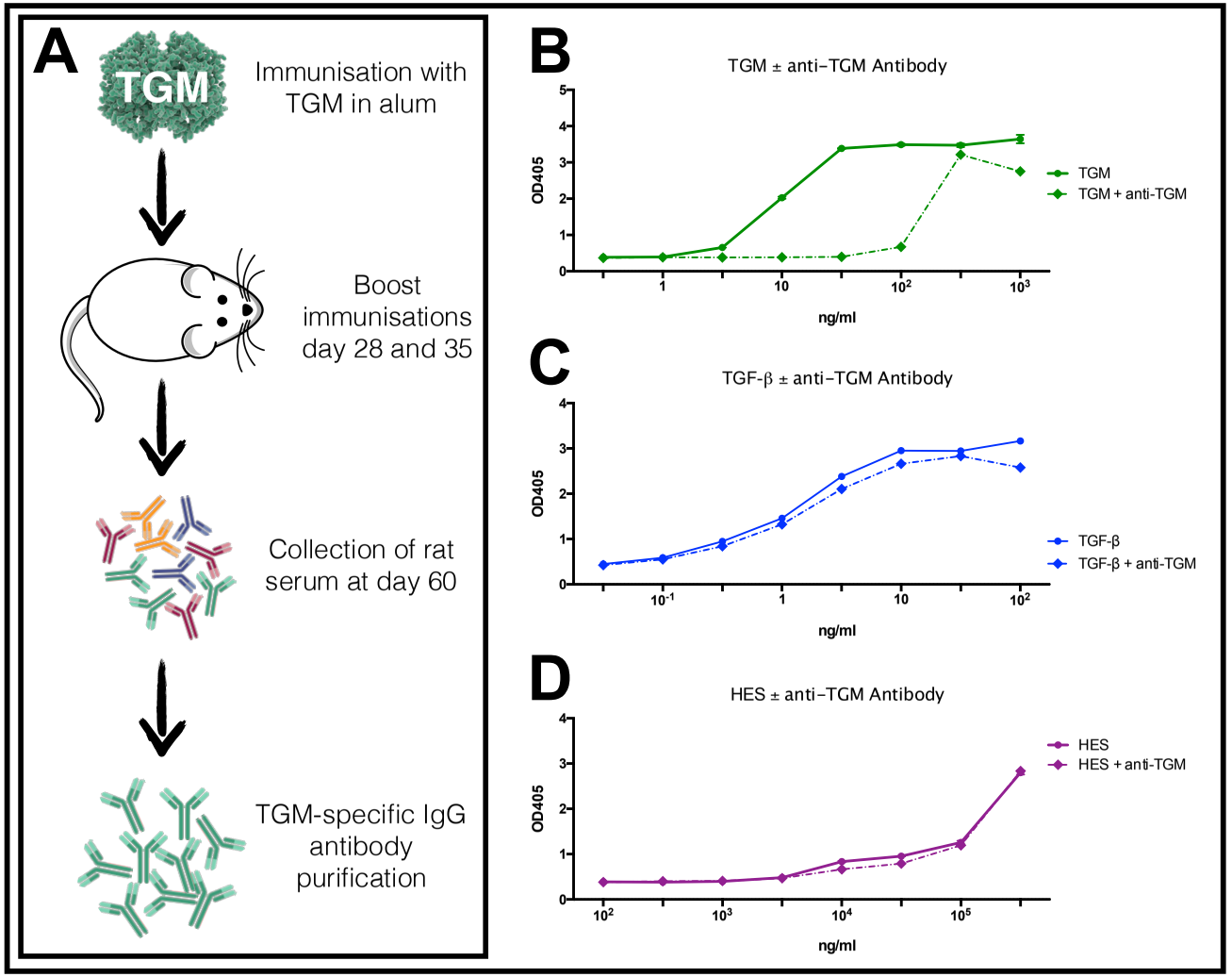


Figure 5-9 Polyclonal anti-TGM antibody neutralises TGM, but not TGF-β or HES
(A) Polyclonocal rat anti-TGM anitbody was generated by immunising rats with 100 μg TGM in Alum on day 0, followed by boost immunisations of 20 μg TGM in alum on day 28 and day 35. Serum was collected on day 60 and TGM-specific IgG was isolated by separation over a protein G column (prepared in advance by Yvonne Harcus). All graphs: activity shown from MFB-F11 bioassay (as described in figure 5-1) after 24 hours of culture at 37°C with: (B) TGF-β and 2.5 μg/ml anti-TGM antibody (interrupted line) or rat IgG control (solid line). (C) TGM with 2.5 μg/ml anti-TGM antibody (interrupted line) or rat IgG control (solid line). (C) HES with 2.5 μg/ml anti-TGM antibody (interrupted line) or rat IgG control (solid line).

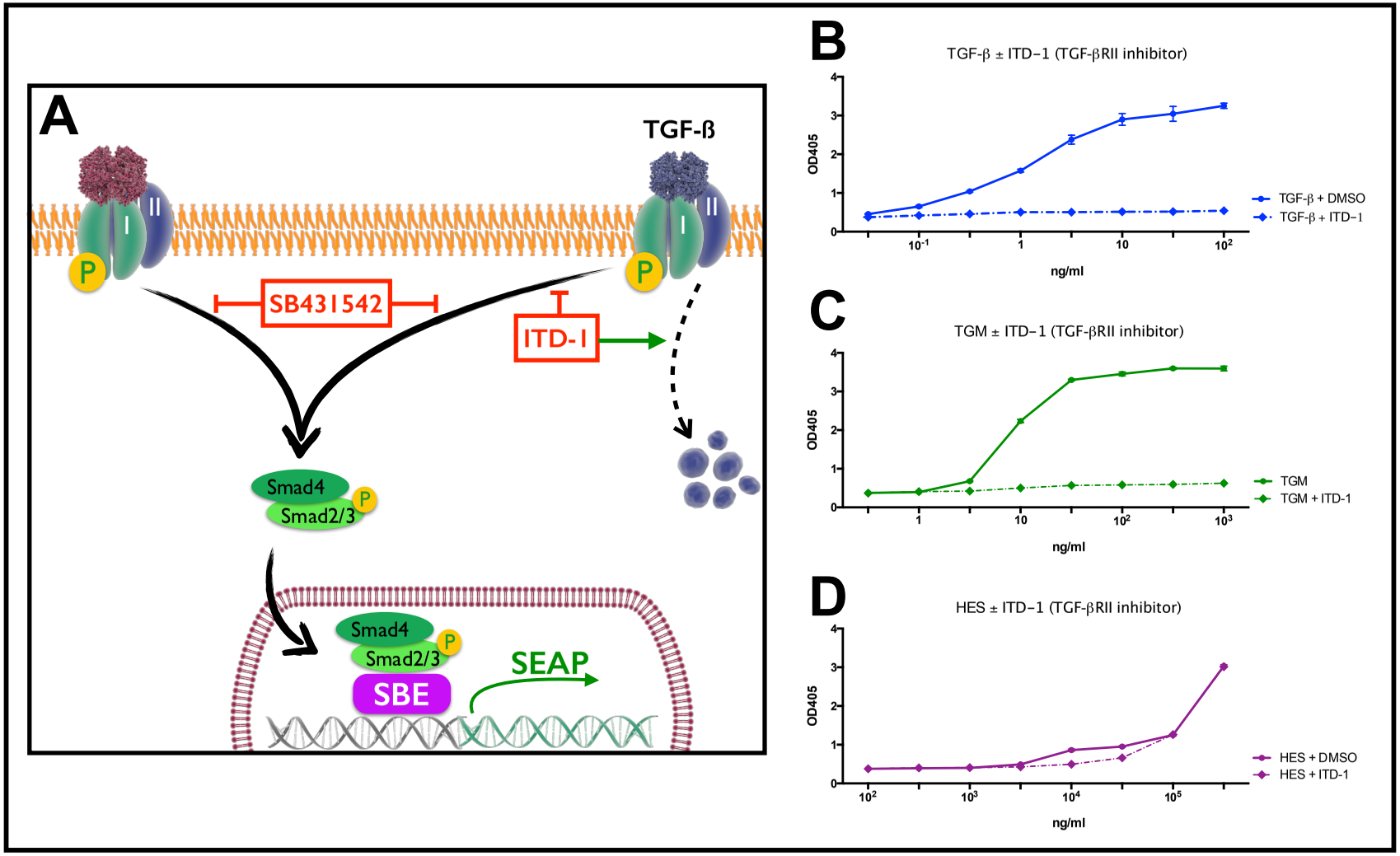


Figure 5-10 TGM and TGF-β signal exclusively via the Type II TGF-β receptor; HES does not
[SEAP - secreted embryonic alkaline phosphatase; SBE - Smad binding element]
(A) Schematic of MFB-F11 TGF-β-responsive bioassay. The small molecule inhibitor, ITD-1 selectively inhibits the TβRII subtype of the Type II TGF-β receptor through enhanced proteolytic degradation, whereas the inhibitor SB431542 inhibits three subtypes of the Type I TGF-β receptor (ALK4, ALK5 and ALK7). All graphs: activity shown from MFB-F11 bioassay (as described in figure 5-1) after 24 hours of culture at 37°C with: (B) TGF-β with 10 µM ITD-1 (interrupted line) or DMSO control (solid line). (C) TGM with10 µM ITD-1 (interrupted line) or DMSO control (solid line). (C) HES with 10 µM ITD-1 (interrupted line) or DMSO control (solid line).

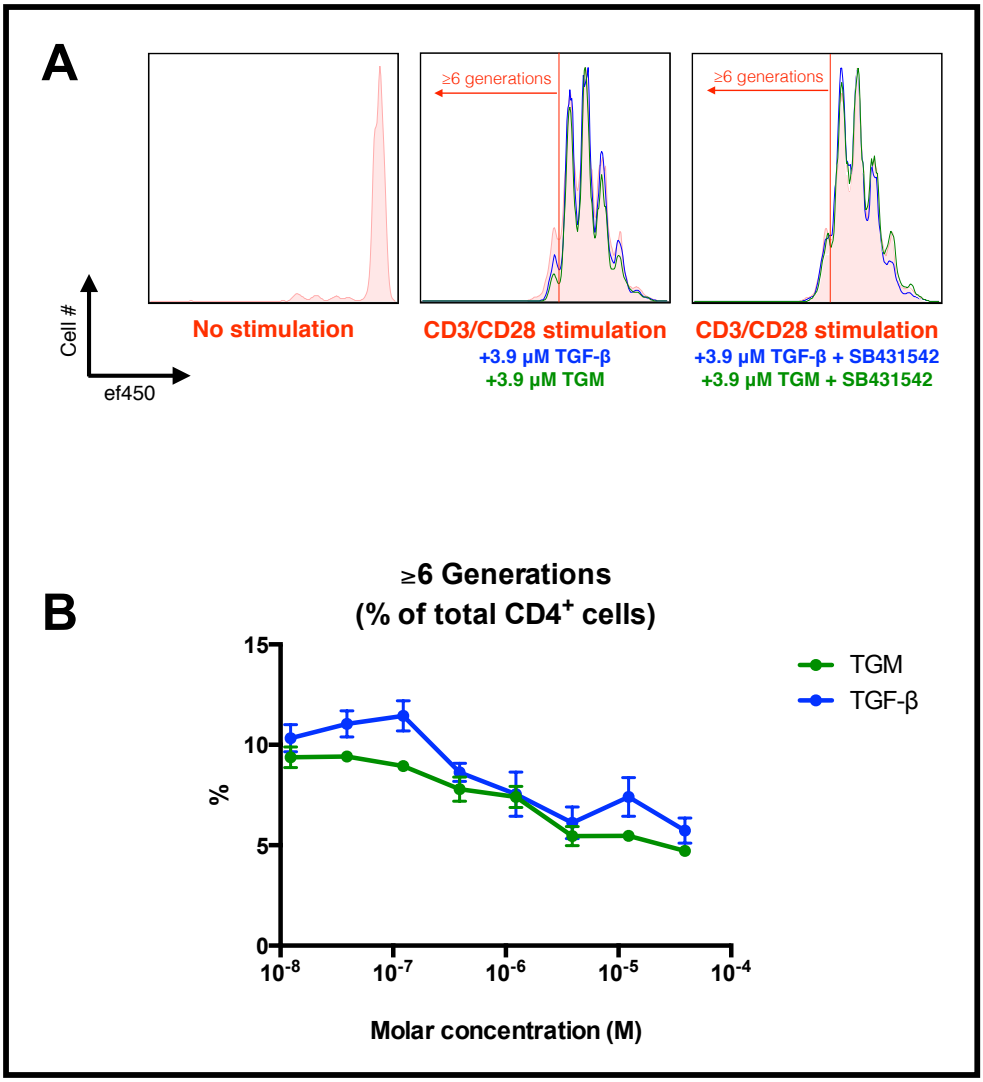


Figure 5-11 TGM inhibits proliferation of human CD4⁺ T cells
Human peripheral blood mononuclear cells were separated from red blood cells over a Ficoll gradient, CD4⁺ T cells were then isolated by MACS positive selection and then labeled with CellTrace® violet for generational tracing. Isolated cells were cultured at 37°C for 96 hours with a 1:1 ratio of CD3/CD28 Dynabeads® and the additional conditions shown.

(A) Overlay histograms showing generational trace of CD4⁺ cells with stimulation only (red), or stimulation with TGF-β (blue) or TGM (green) at the concentrations shown, with or without stimulation with the Type I TGF-β receptor inhibitor, SB431542.

(B) Percentage of CD4⁺ cells shown in (A) that have proliferated to six generations or more, plot against molar concentration of TGM or TGF-β.

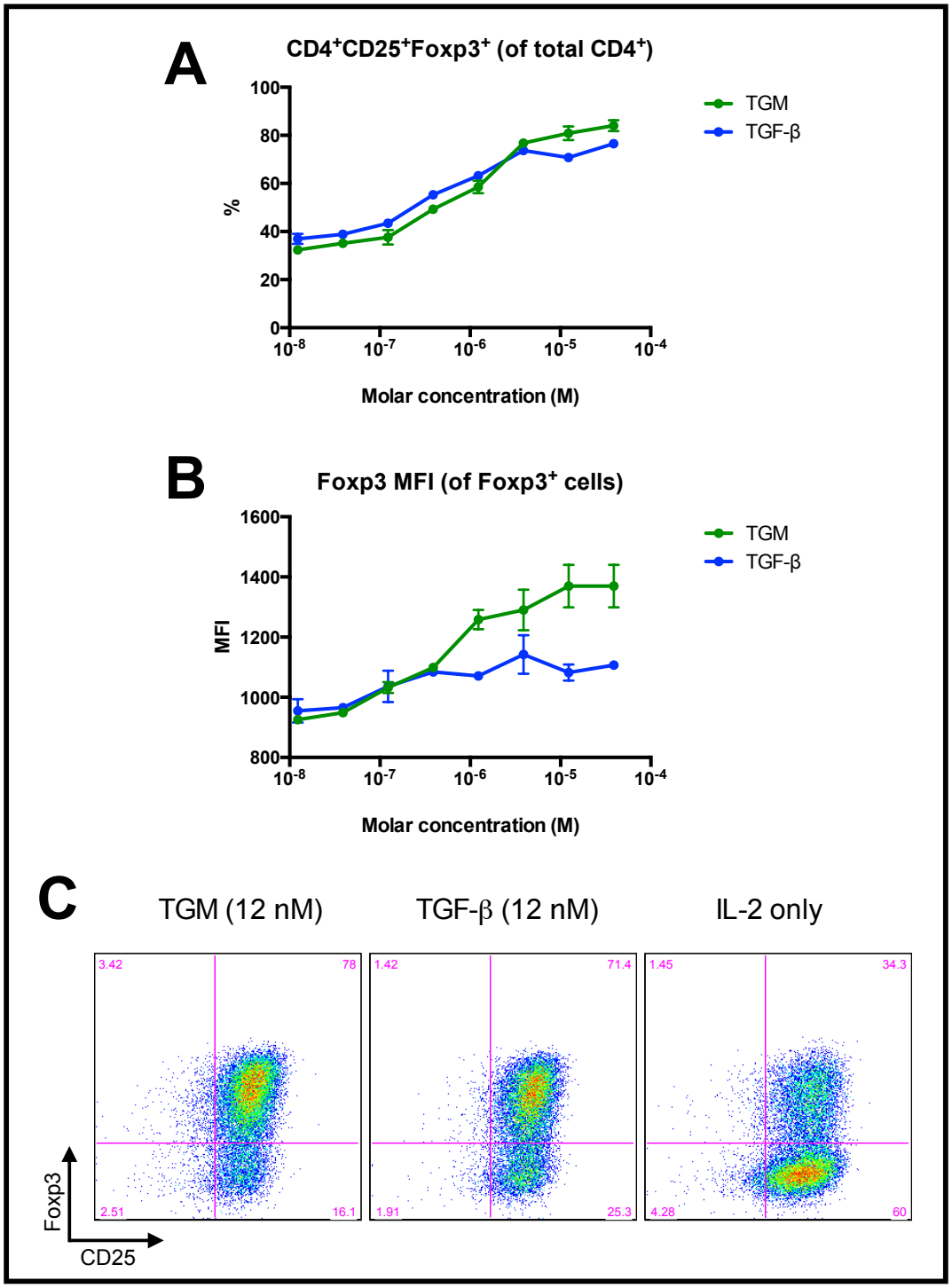


Figure 5-12 TGM induces Foxp3 expression in human CD4+ T cells
Human peripheral blood mononuclear cells were separated from red blood cells over a Ficoll gradient and CD4+ T cells were then isolated by MACS positive selection. Isolated cells were cultured at 37°C for 96 hours with a 1:1 ratio of CD3/ CD28 Dynabeads® and variable concentrations of TGF-β or TGM.

(A) Treg induction (expressed as a percentage of total CD4+ cells against molar concentration of TGM or TGF-β)

(B) Mean fluorescence intensity of Foxp3 expression (of Foxp3+ cells) against molar concentration of TGM or TGF-β.

(C) Representative flow cytometry plots (CD4 population shown).

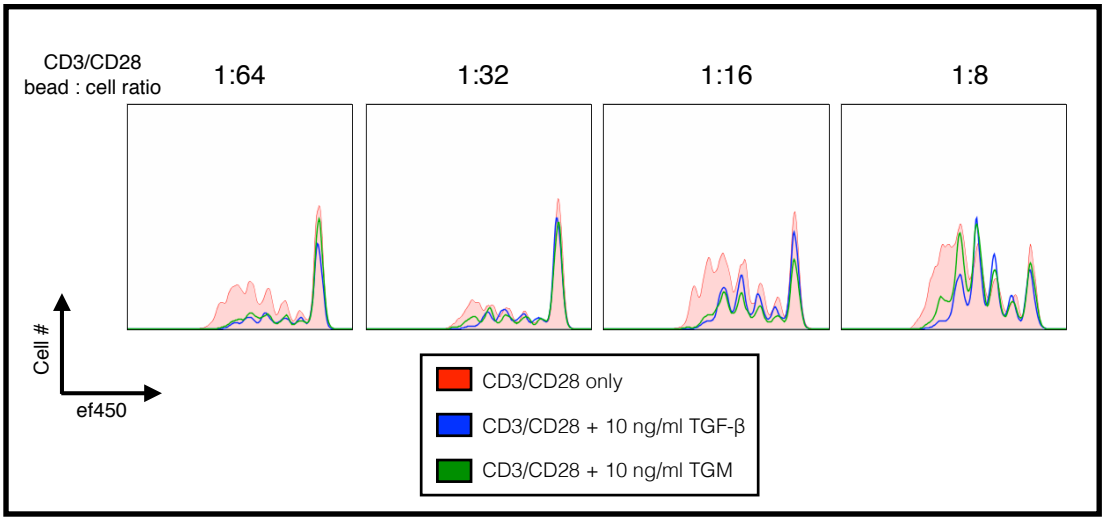


Figure 5-13 TGM suppresses human CD8$^+$ T cell proliferation
CD8+ T cells isolated from human peripheral blood by ficoll gradient separation followed by positive MACS selection. Cells were cultured for 96 hours with variable concentrations of CD3/CD8 Dynabeads® with or without 10 ng/ml TGM or TGF-β (as shown). Histograms of cell division, as assessed by generational tracing with CellTrace® violet.

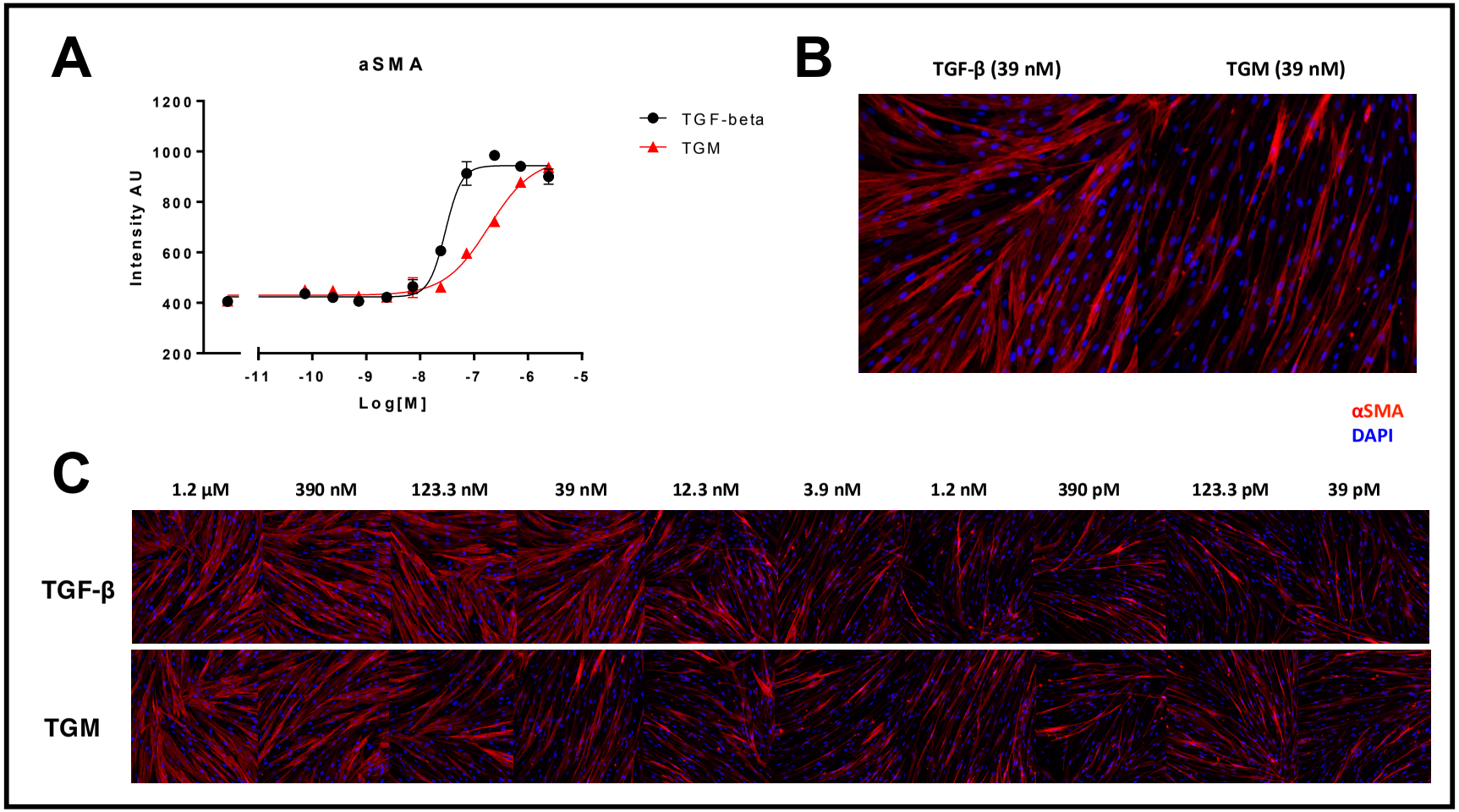


Figure 5-14 TGM induces alpha smooth muscle actin production by human lung fibroblasts less potently than TGF-β
Macromolecular crowding assay of WI-38 human lung fibroblasts (performed by Dr Hannah Woodcock, Chambers laboratory, UCL). Fibroblasts were cultured under crowded conditions for 48 hours at 37°C in DMEM supplemented by 0.4% FCS and variable concentrations of TGM or TGF-β as shown. At the end of the culture period, cells were fixed with methanol and stained for α-smooth muscle actin (AF594) and counterstained with DAPI. Integrated morphology analysis was then used to quantify the area of fluorescent α-SMA staining. (A) Comparative intensity of α-SMA staining against molar concentration of TGM / TGF-β. (B) Representative staining (39 nM). (C) Representative staining (all concentrations).

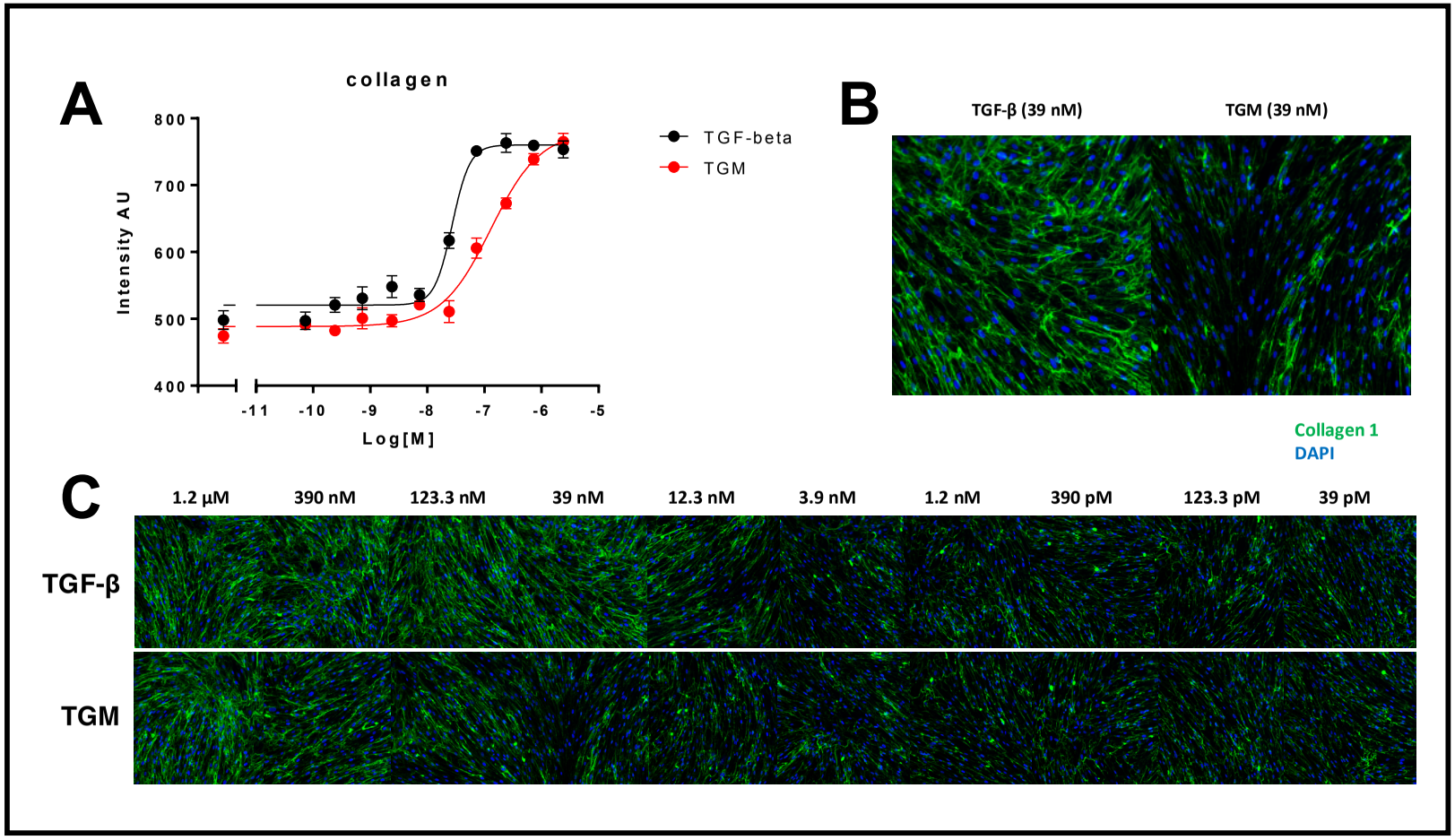


Figure 5-15 TGM induces type I collagen production by human lung fibroblasts less potently than TGF-β
Macromolecular crowding assay of WI-38 human lung fibroblasts (performed by Dr Hannah Woodcock, Chambers laboratory, UCL). Fibroblasts were cultured under crowded conditions for 48 hours at 37°C in DMEM supplemented by 0.4% FCS and variable concentrations of TGM or TGF-β as shown. At the end of the culture period, cells were fixed with methanol and stained for type I collagen (AF488) and counterstained with DAPI. Integrated morphology analysis was then used to quantify the area of fluorescent type I collagen staining. (A) Comparative intensity of type I collagen staining against molar concentration of TGM / TGF-β. (B) Representative staining (39 nM). (C) Representative staining (all concentrations).

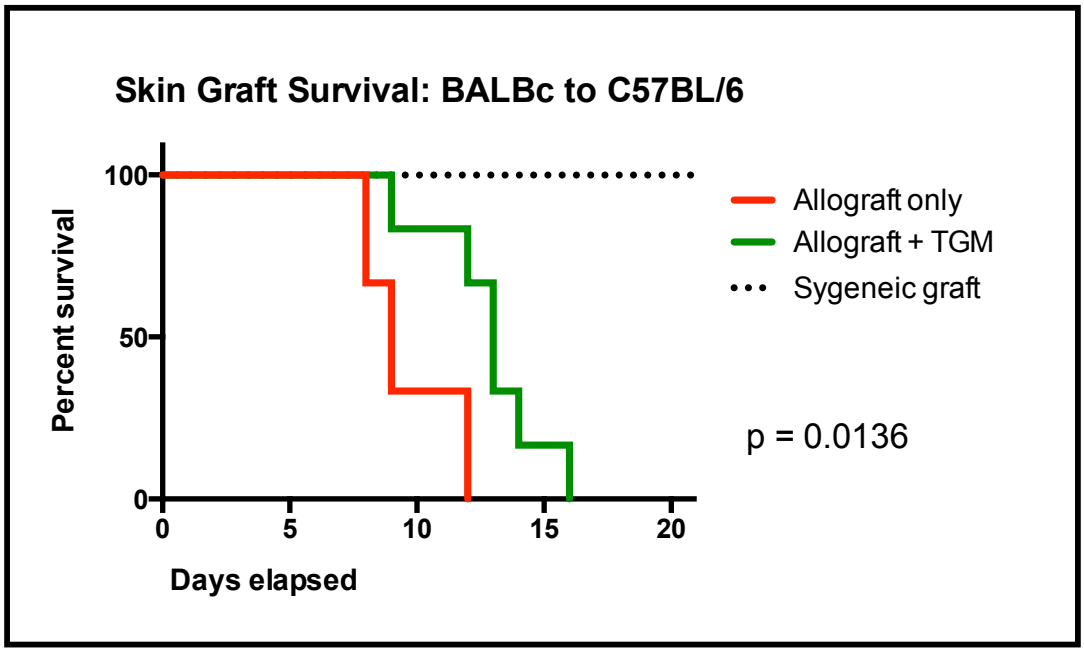


Figure 5-16 TGM prolongs survival of fully-allogeneic skin grafts
Kaplan-Meier curve of full-thickness skin graft survival: allograft only (BALB/c to C57BL/6 skin graft, n = 6), allograft + TGM (BALB/c to C57BL/6 skin graft immediately preceded by implantation of an intraperitoneal osmotic minipump eluting 79.2 ng/day of TGM, n = 6) and syngeneic grafts (C57BL/6 to C57BL/6 skin graft controls, n = 3). Mantel-Cox comparison of allograft vs. allograft + TGM survival curves: p = 0.0136.

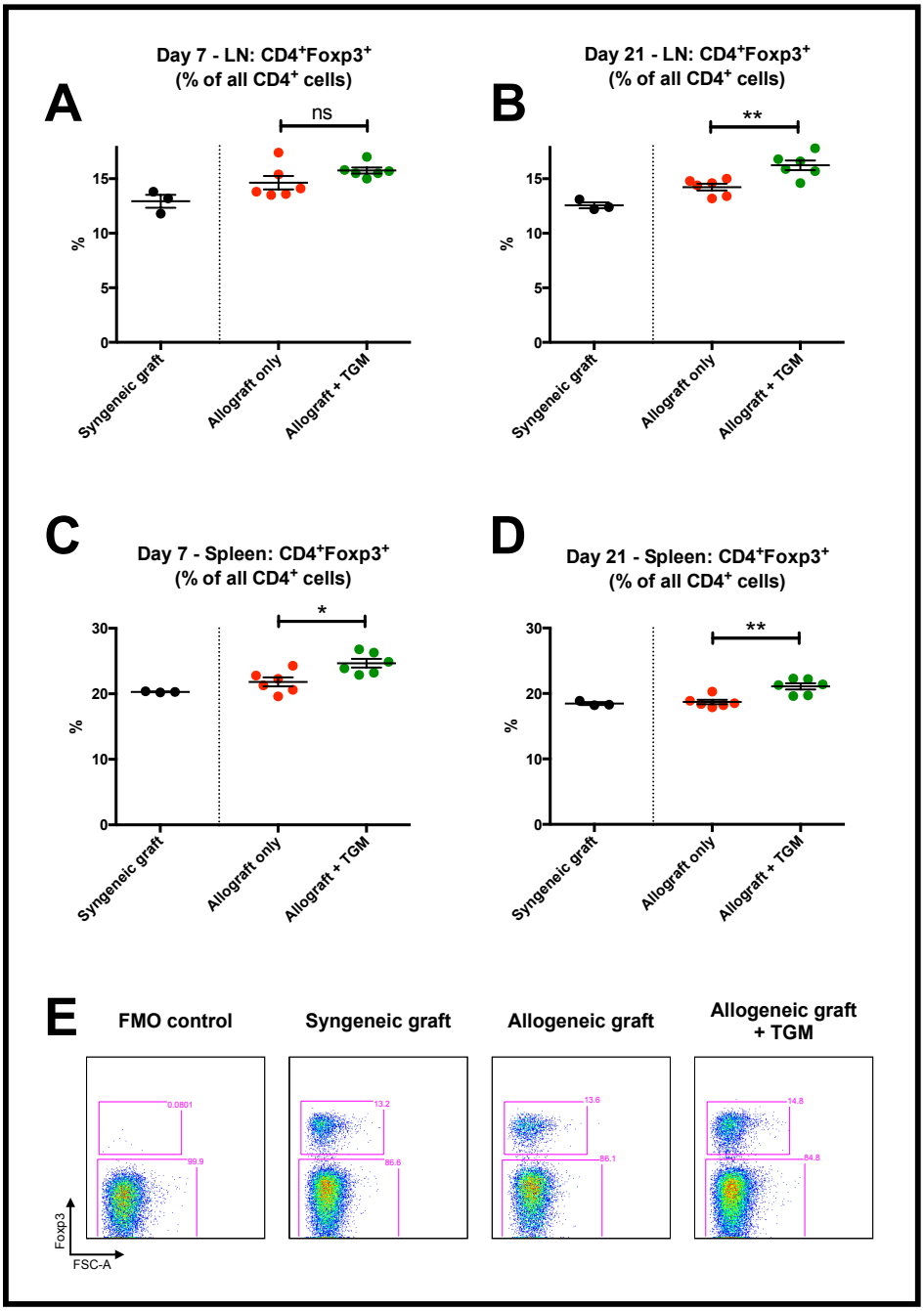


Figure 5-17 TGM induces Foxp3 expression by CD4$^+$ T cells *in vivo*
Full-thickness allogeneic (BALB/c to C57BL/6) and syngeneic control (C57BL/6 to C57BL/6) skin grafts were performed with the following experimental conditions: allograft only or allograft + TGM (allograft immediately preceded by implantation of an intraperitoneal osmotic minipump eluting 79.2 ng/day of TGM). Two independent experiments were performed to allow for analysis of splenic and allograft draining lymph node CD4$^+$ T cell populations at 7 and 21 days after transplantation. (A) Percentage of CD4$^+$ T cells within the allograft draining lymph node expressing Foxp3 7 days after transplantation. (B) Percentage of CD4$^+$ T cells within the allograft draining lymph node expressing Foxp3 21 days after transplantation. (C) Percentage of CD4$^+$ T cells within the spleen expressing Foxp3 7 days after transplantation. (D) Percentage of CD4$^+$ T cells within the spleen expressing Foxp3 21 days after transplantation. (E) Representative flow cytometry plots from (B).

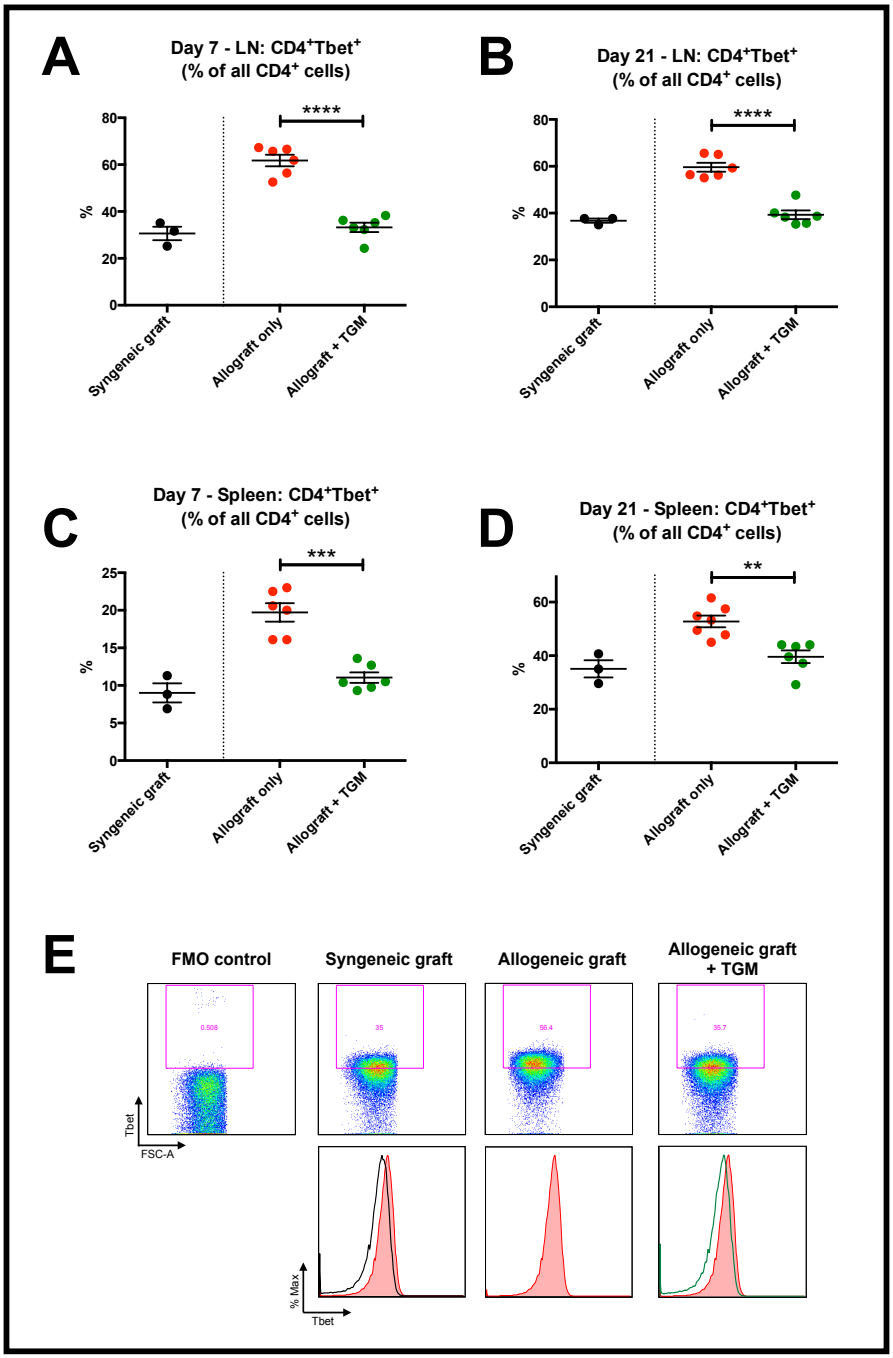


Figure 5-18 TGM suppresses expression of Tbet by CD4$^+$ T cells
Full-thickness allogeneic (BALB/c to C57BL/6) and syngeneic control (C57BL/6 to C57BL/6) skin grafts were performed with the following experimental conditions: allograft only or allograft + TGM (allograft immediately preceded by implantation of an intraperitoneal osmotic minipump eluting 79.2 ng/day of TGM). Two independent experiments were performed to allow for analysis of splenic and allograft draining lymph node CD4$^+$ T cell populations at 7 and 21 days after transplantation. (A) CD4$^+$Tbet$^+$ cells as a percentage of all CD4$^+$ cells within the allograft draining lymph node 7 days after transplantation. (B) CD4$^+$Tbet$^+$ cells as a percentage of all CD4$^+$ cells within the allograft draining lymph node 21 days after transplantation. (C) CD4$^+$Tbet$^+$ cells as a percentage of all splenic CD4$^+$ 7 days after transplantation. (D) CD4$^+$Tbet$^+$ cells as a percentage of all splenic CD4$^+$ 21 days after transplantation. (E) Representative flow cytometry plots and histograms from (B), red shaded area indicates the Tbet expression of the allograft only group.

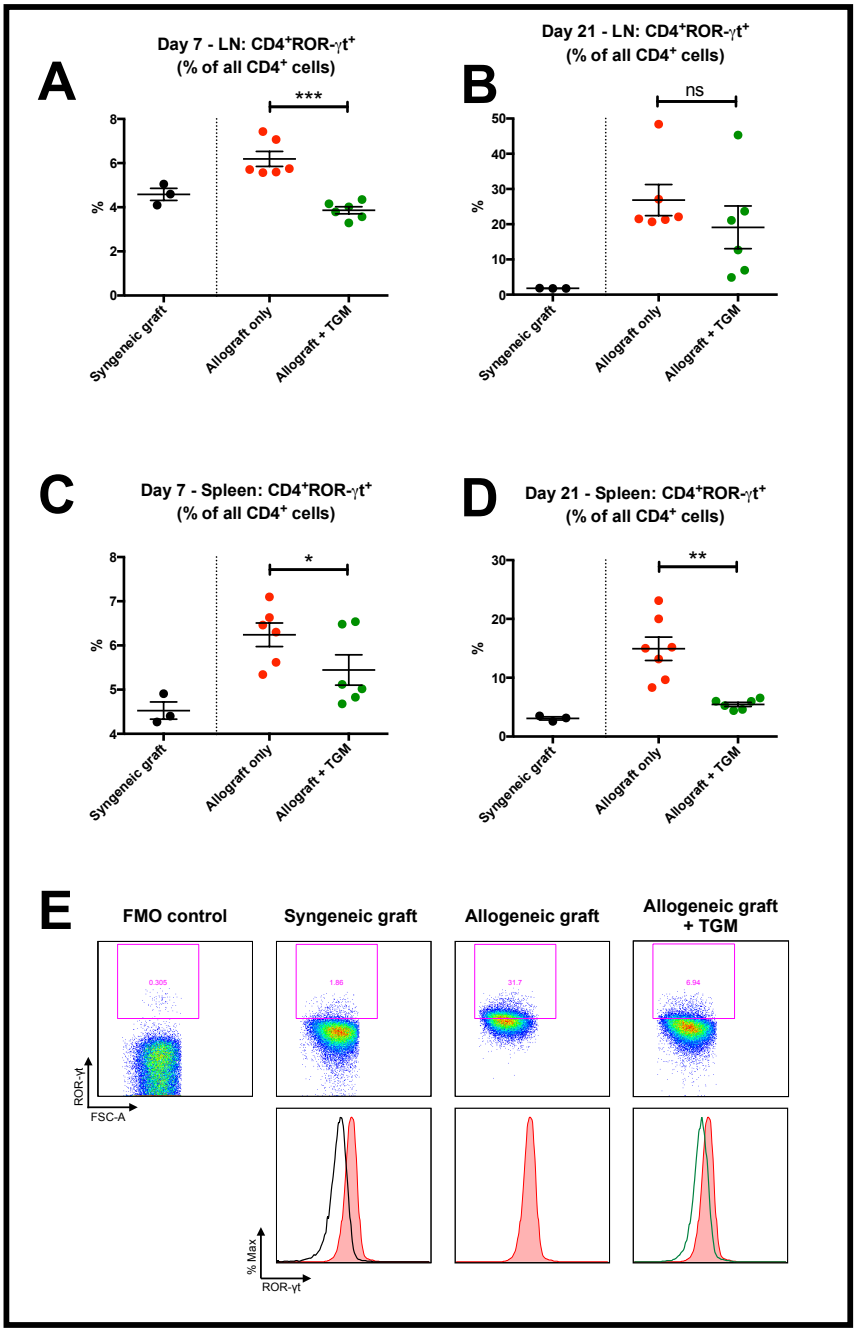


Figure 5-19 TGM suppresses expression of ROR-γt by CD4$^+$ T cells
Full-thickness allogeneic (BALB/c to C57BL/6) and syngeneic control (C57BL/6 to C57BL/6) skin grafts were performed with the following experimental conditions: allograft only or allograft + TGM (allograft immediately preceded by implantation of an intraperitoneal osmotic minipump eluting 79.2 ng/day of TGM). Two independent experiments were performed to allow for analysis of splenic and allograft draining lymph node CD4$^+$ T cell populations at 7 and 21 days after transplantation. (A) CD4$^+$ROR-γt$^+$ cells as a percentage of all CD4$^+$ cells within the allograft draining lymph node 7 days after transplantation. (B) CD4$^+$ROR-γt$^+$ cells as a percentage of all CD4$^+$ cells within the allograft draining lymph node 21 days after transplantation. (C) CD4$^+$ROR-γt$^+$ cells as a percentage of all splenic CD4$^+$ 7 days after transplantation. (D) CD4$^+$ROR-γt$^+$ cells as a percentage of all splenic CD4$^+$ 21 days after transplantation. (E) Representative flow cytometry plots and histograms from (B), red shaded area indicates the ROR-γt expression of the allograft only group.

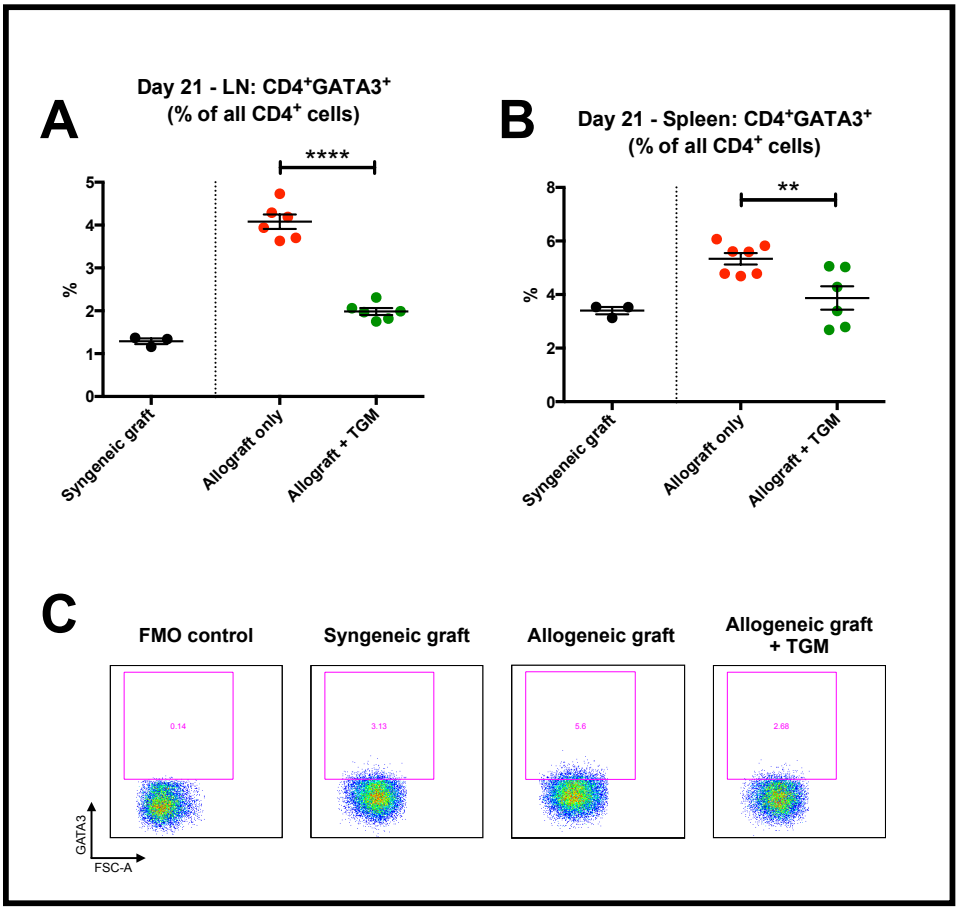


Figure 5-20 TGM suppresses expression of GATA3 by CD4$^+$ T cells

(A) CD4$^+$ T cells from the allograft draining (inguinal) lymph node of C57BL/6 mice 21 days after transplantation of a full-thickness BALB/c skin graft or syngeneic C57BL/6 skin graft control. Experimental groups: allograft only (BALB/c to C57BL/6 skin graft, n = 6), allograft + TGM (BALB/c to C57BL/6 allograft immediately preceded by implantation of an intraperitoneal osmotic minipump eluting 79.2 ng/day of TGM, n = 6). CD4$^+$GATA3$^+$ cells as a percentage of total CD4$^+$ cells shown.

(B) CD4$^+$ T cells from the spleen of the animals described in (A). CD4$^+$GATA3$^+$ cells as a percentage of total CD4$^+$ cells shown.

(C) Representative flow cytometry plots (GATA3$^+$ gating of CD4$^+$ cells shown, from splenic populations in (B)).

Statistical significance assessed by two-tailed t tests. FMO - fluorescence minus one.

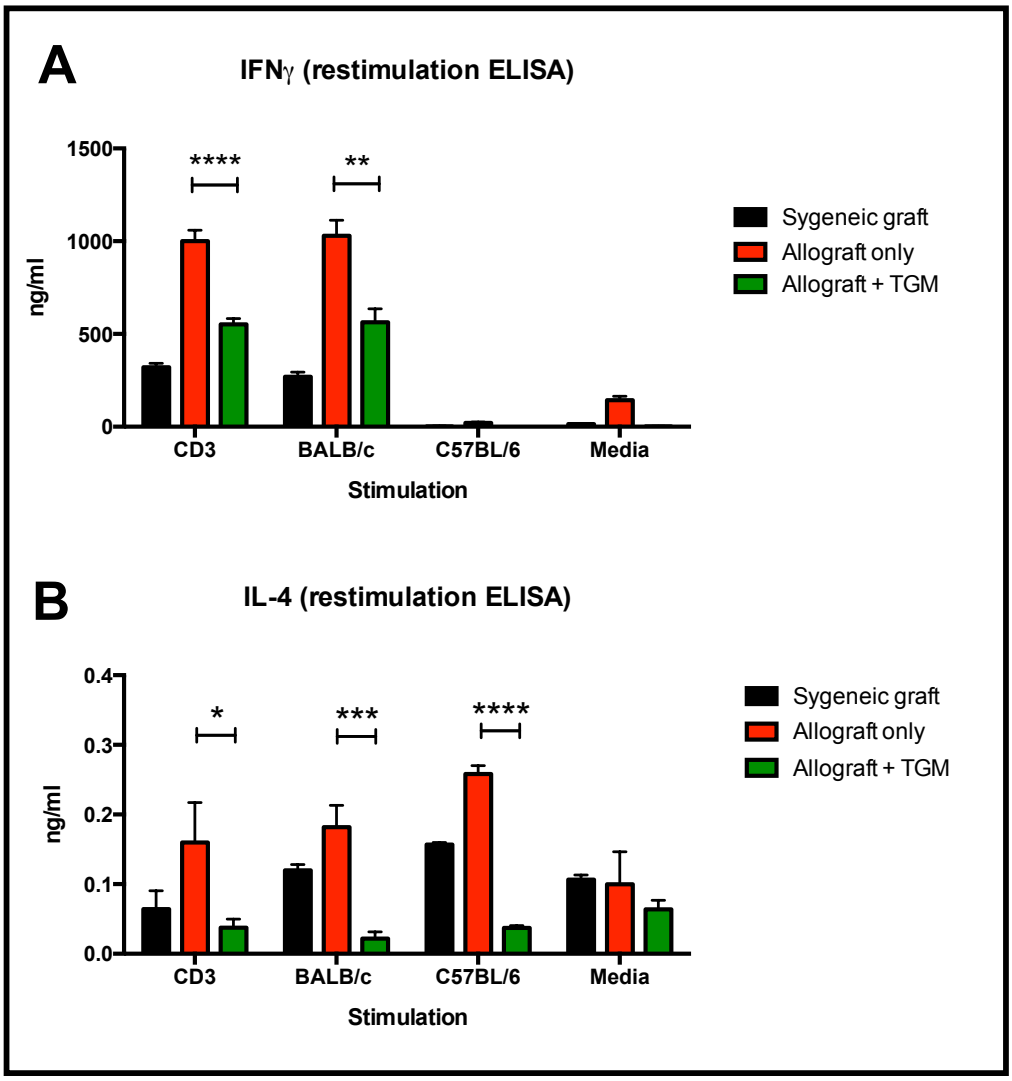


Figure 5-21 TGM suppresses IFNγ and IL-4 production upon restimulation
Splenocytes were harvested from C57BL/6 mice 21 days after transplantation of a full-thickness BALB/c skin graft or syngeneic control graft. Experimental groups were: allograft only and allograft + TGM (allograft immediately preceded by implantation of an intraperitoneal osmotic minipump eluting 79.2 ng/day of TGM). Isolated splenocytes (10^6) were restimulated *ex-vivo* for 72 hours at 37°C in duplicate with the following conditions (from left): soluble CD3 (1 μg/ml), 10^6 irradiated BALB/c (donor strain alloantigen) splenocytes, 10^6 irradiated CBA (third party strain alloantigen) splenocytes, 10^6 irradiated C57BL/6 (recipient strain syngeneic antigen) splenocytes, or complete RPMI media. (A) Restimulation supernatant IFNγ concentration measured by ELISA. (B) Restimulation supernatant IL-4 concentration measured by ELISA. Statistical significance assessed by two-tailed, unpaired t tests.

5.3 Discussion

The experimental results described in this chapter reveal that TGM not only mimics the action of TGF-β, but also exhibits a number of properties that promote it as a more therapeutically tractable mediator than TGF-β itself.

Despite ligating the TGF-β receptor complex, TGM is structurally distinct from the TGF-β molecule - it shares no sequence homology with TGF-β, is almost four times greater in size and is not recognised by pan-vertebrate anti-TGF-β antibodies. This in itself could be of significant therapeutic relevance – *in vitro*, TGM has been shown to stimulate enhanced downstream signalling compared to TGF-β (as evidenced by elevated MFB-F11 signal, Smad2 phosphorylation and Foxp3 expression). The mechanism behind this effect is currently unknown, but may be as a result of differing receptor binding kinetics or of TGM failing to ligate pseudoreceptors such as BAMBI[327] on the cell surface. *In vivo*, TGM is constitutively active – unlike TGF-β, it has no requirement for enzymatic cleavage and may evade further regulatory mechanisms such as the binding of 'ligand trap' proteins, which would allow for therapeutic dose-response titration that is not possible with exogenous TGF-β.

Grainger *et al* previously demonstrated that blockade of TGF-β signalling boosts immunity to *H. polygyrus* infection (as demonstrated by reductions in worm burden), whilst antibody-mediated neutralisation of circulating host TGF-β has no effect[233]. Thus, it seems that parasite-derived TGF-β activity plays a vital role in the immunomodulation required for successfully establishing chronic infection and it is possible that TGM is the key mediator involved in this process. Figure 5-9 shows that a polyclonal antibody to recombinant TGM neutralises the MFB-F11 signal of TGM but not HES. If TGF-β activity is evolutionarily crucial, TGM may belong to a family of products, each of which are individually redundant - the other homologues of TGM that have been identified within HES may exhibit as much, or greater, TGF-β-like activity. Failure to neutralise HES by anti-TGM antibody may instead be due to antibody binding of extraneous epitopes in recombinant TGM arising from the pSecTag expression vectors (e.g. His-tag or Myc-tag) that are not present in naturally produced TGM within HES. Western blots of HES probed with anti-TGM antibody

failed to answer this question due to extensive non-specific binding of control IgG to HES proteins (data not shown). However, selective blockade of the TβRII subtype of the type II TGF-β receptor family (with the inhibitor ITD-1) completely blocked the MFB-F11 signal arising from TGM and TGF-β, but not HES (Figure 5-10). This strongly suggests that additional TGF-β-like homologues are present within HES and signal via a different heteromeric combination of type I and type II TGF-β receptors. Expression of the additional known TGM homologues is currently underway.

The novel finding that TGM induces elevated expression of Foxp3 compared to the maximum achieved with TGF-β in both murine (Figure 5-5) and human (Figure 5-12) $CD4^+$ T cells may have a number of implications. In terms of human Treg induction, further optimisation of *in vitro* assays is required to definitively assess the potential of TGM – the assay described in Figure 5-12 assessed Treg composition of a whole $CD4^+$ population in culture and therefore the Treg expansion seen with TGM (and TGF-β) is likely a combination of both *de novo* Foxp3 expression in induced pTreg and proliferation of tTreg. Further experiments are needed with naïve $CD4^+$ cells ($CD4^+CD25^-CD62L^{hi}$ isolated by FACS), lower levels of CD3/CD28 stimulation and addition of exogenous IL-2 to culture media.

Intensity of Foxp3 expression by Treg has previously been shown to directly correlate with suppressive ability[333]. The suppression assay described in Figure 5-8 showed TGM-induced Treg to be equal to TGF-β-induced Treg in terms of suppressive ability, but this *in vitro* assay may be insufficiently sensitive to detect subtle differences – further investigation with *in vivo* models is required. High concentrations of TGF-β are known to promote Treg over Th17 differentiation[342]. TGM appears to be a more potent ligand than TGF-β and may therefore tip the balance away from Th17 differentiation; suppression of ROR-γt expression by TGM in allogeneic skin graft recipients supports this (figure xx). Finally, further investigation is required to determine whether enhanced intensity of Foxp3 expression promotes stability of the Treg phenotype, particularly in the presence of inflammatory cytokines such as IL-6.

In the context of wound healing, enhanced collagen deposition could be potentially beneficial during the course of *H. polygyrus* infection. It is possible that TGM plays a role in repairing the intestinal damage caused by *H. polygyrus* migrating through to the subserosal layer, but this is unlikely because TGM suppresses Th2 cytokines (which are fundamentally required for effective wound healing in helminth infection[370]) and, in direct contrast to induction of Foxp3, TGM is less efficacious than TGF-β in inducing myofibroblast differentiation as demonstrated by alpha smooth muscle actin expression and collagen deposition (Figures 5-14 and 5-15).

Whilst HES contains numerous immunomodulatory mediators in addition to TGM that likely act to some degree synergistically, recombinant TGM offers several advantages including scalable production, a definable mechanism of action and the opportunity for modification to reduce immunogenicity and optimise pharmacokinetic characteristics for pharmacological use.

Furthermore, combination of TGM with currently available immunomodulatory agents may be a viable future therapeutic strategy. In particular, combining rapamycin or retinoic acid with the potent and predictable TGF-β activity of TGM could help to promote Treg over Th17 differentiation. Compared to TGF-β, TGM has been shown to be more efficacious in inducing Foxp3 and less so at inducing myofibroblast differentiation – this relationship is particularly encouraging for the possibility of a tolerogenic therapy with a larger therapeutic window than is currently available.

6 Final Discussion

The development of organ transplantation has revolutionised the treatment of numerous diseases, transforming and extending the lives of millions of patients worldwide. However, the almost ubiquitous necessity for life-long immunosuppression remains problematic. A considerable proportion of the precious resource of transplanted organs is lost to rejection every year[371]. Further, patients are exposed to an array of serious adverse effects that are frequently life-limiting[141]. Until recently, the decision to accept this therapeutic risk profile has been straightforward, given the alternative of imminently life-threatening organ failure or the significant risk of mortality with dialysis[15]. However, the advent of hand, face and other non-life-extending transplants has posed a conspicuous challenge to the principle of nonmalificence in this regard and highlighted the need for novel alternatives to currently employed immunosuppression regimens[372].

This thesis has explored the potential of *Heligmosomoides polygyrus* and its secreted products to counteract allo- and autoimmune responses. Whilst the overarching focus has been on transplantation, study of the autoimmune model, EAE, has provided some key additional insights. Many of the currently available immunosuppressant agents are utilised in combating both allograft rejection and autoimmune conditions, and it is anticipated that emerging novel therapeutic agents could be of benefit in both clinical settings also.

Chapter 3 began by demonstrating for the first time that infection with *H. polygyrus* is capable of prolonging the viability of a transplanted allograft. This effect was then recapitulated by administration of HES, confirming that allograft protection is resulting from the action of a soluble mediator (or mediators) secreted by the helminth; its physical presence (i.e. live infection) is not necessary. Further experiments demonstrated that infection with *H. polygyrus* polarised alloimmune Th1 and Th17 responses towards a Th2 phenotype. HES suppressed Th1 and Th17 differentiation but did not induce allograft-specific Th2 responses. Contrary to the

proposed mechanism of allograft protection afforded by other helminth species[267,269,270], Th2 polarisation was shown to be unnecessary, as demonstrated by protection of skin grafts in allogeneic transplant combinations of IL-4Rα-deficient mice by administration of HES.

Chapter 4 explored the ability of *H. polygyrus* and HES to ameliorate disease in the experimental model of multiple sclerosis, EAE. Whilst treatment with HES led to a delayed onset of disease, *H. polygyrus* was dramatically more effective, reducing the incidence, duration and maximal severity of disease. In contrast to the situation with alloimmunity, a considerable degree of the protection from EAE afforded by *H. polygyrus* infection was found to be Th2-dependent. IL-4Rα$^{-/-}$ mice treated with *H. polygyrus* infection experienced a delayed onset of EAE, but no reduction in the maximal severity of disease. This effect was very similar to that of wild-type animals treated with HES. Analysis of CD4^{+} T cell populations revealed that both *H. polygyrus* and HES promote expression of PD-1 and ST2 on the surface of Foxp3^{-} effector T cells. Further assessment of the effects of *H. polygyrus* and HES by adoptive transfer of antigen-specific DO11.10 CD4^{+} T cells and immunisation with cognate antigen demonstrated that both treatments could effectively promote expansion of Foxp3^{+} Treg and suppress proliferation of the Foxp3^{-} effector T cell population.

Chapter 5 adopted a more reductionist approach to examine the cellular mechanism of action and functional significance of the newly identified immunomodulatory molecule within HES, TGM. Despite sharing no sequence homology with TGF-β, TGM was found to act via the TGF-β receptor complex to induce Foxp3 expression and suppress proliferation in both murine and human T cells. The unique structure of TGM compared to TGF-β was confirmed by demonstration of mutually exclusive neutralisation by respective specific antibodies. Important functional differences were also identified: TGM achieves a greater intensity of downstream signalling from the TGF-β receptor complex than TGF-β itself, and this relationship strongly correlates with the corresponding efficacy of TGM compared to TGF-β in induction of Foxp3 expression by CD4^{+} T cells. When TGM was assessed for its pro-fibrotic potential by culture with human lung fibroblasts, the opposite relationship was

observed: TGM can induce fibrosis, but is less potent is this respect than equivalent concentrations of TGF-β. Finally, the functional capability of TGM to ameliorate allograft rejection was assessed *in vivo*. TGM was found to significantly prolong the survival of fully-allogeneic skin grafts and analysis of the $CD4^+$ T cell populations of allograft recipients revealed significant induction of $Foxp3^+$ Treg in addition to significant suppression of Th1, Th2 and Th17 effector phenotypes.

The data outlined above provide some novel insights into previous observations made in other experimental models. The vigorous Th2 immune response generated by *H. polygyrus* infection has been long recognised[373] and, more recently, it has been shown that HES might contribute to this effect (Th2 responses can be generated by adoptive transfer of DCs that have been exposed to HES)[374]. However, the relative contribution of the dual stimuli of HES and intestinal trauma that are present in *H. polygyrus* infection has not been previously established.

The observation presented here that infection with *H. polygyrus* larvae induces a strong Th2 response, but treatment with a continuous infusion of HES over the same timeframe does not, indicates that the dominant stimulus for Th2 immunity *in vivo* is related to the physical presence of the parasite (likely as a result of larval migration to the intestinal submucosa). Indeed, whereas *H. polygyrus* infection was found to significantly increase expression of GATA3 in the splenic $CD4^+$ T cell populations of allograft recipients, both HES and TGM were shown to significantly suppress expression even compared to untreated allograft recipients. This finding reflects the impact of HES during a rapidly evolving alloimmune response *in vivo*, within a short timeframe and with no period of pre-treatment. It is not incongruous with the previously published ability of HES-treated DCs to drive Th2 immune responses[374]. In fact, this possibly explains the reported necessity of a period of pretreatment to successfully ameliorate EAE with soluble products from other parasite species (such as *S. mansoni*[375] and *T. spiralis*[226,227]), in contrast to treatment with *H. polygyrus* infection, which can considerably ameliorate disease without any pretreatment, or even precipitate improvement in the clinical condition of animals with established disease[218]: generation of a Th2 response by soluble mediators indirectly via DCs takes time and can only influence the disease course if the DC phenotype has been

successfully altered prior to antigen presentation and generation of the T cell response. By contrast, live infection generates a powerful Th2 response directly as a result of intestinal trauma and can therefore suppress (through cross regulation) the established Th1/Th17 $CD4^+$ T cells mediating EAE. Further investigation with passive models of EAE (adoptive transfer of antigen-specific T cells that have been polarised to a Th1 phenotype *in vitro*) could test this hypothesis by bypassing the influence of dendritic cells during antigen presentation.

It is also clear that HES can induce a number of regulatory mechanisms in the absence of a Th2 response including enhanced PD-1 expression, expansion of $Foxp3^+$ Treg and suppression of Th1 and Th17 differentiation. The functional significance of these effects appears to vary considerably with the specific disease model under investigation: HES appears to be similarly effective to *H. polygyrus* in protecting transplanted allografts from rejection, but much less so in ameliorating EAE.

Further work to characterise the mechanism behind HES and TGM-mediated suppression of Th1, Th2 and Th17 effector cell phenotypes is required – this effect could be simply as a result of the ongoing presence of the expanded Treg population, or alternatively because of changes to the alloreactive $CD4^+Foxp3^-$ T cell population itself (similar to that which is evident by the donor alloantigen-specific 'Th2 switch' in the cytokine profile of splenocytes from allograft recipients infected with *H. polygyrus*). This could be investigated by isolating the $CD4^+Foxp3^-$ splenic T cell populations of allograft recipients prior to allogeneic restimulation for cytokine profile analysis, and by assessing the impact of HES and TGM *in vivo* following Treg depletion.

When considering the translational potential of TGM and other parasite products, non-Th2 dependent mechanisms are likely have the greatest translational potential, even if the magnitude of the effect observed in experimental murine models is less than that of infection with *H. polygyrus* larvae. The current pharmacopoeia of immunosuppressive agents for transplantation is effective in suppressing acute episodes of rejection and enhancing allograft survival in the short term, but relatively

deficient in the ability to suppress Th17 responses and to prevent long-term graft dysfunction. Whilst induction of a Th2 response might enable additional suppression of pathological immune responses through cross-regulation (manifest as more effective amelioration of acute disease in animal models such as EAE), in the long term, alloantigen-specific Th2 responses may be detrimental to allograft function[71] (due to promotion of interstitial fibrosis) and thereby exacerbate the current clinical problem rather than improve it. By contrast, TGM effectively suppresses both Th17 and Th2 differentiation and may therefore represent exactly the type of agent that is needed to improve long-term graft function, even if it initially needs to be co-administered with additional synergistic immunosuppressive agents in the immediate post-transplantation period.

The striking manipulation of alloantigen-specific T cell responses achieved by HES and TGM (as demonstrated by restimulation assays) also presents another interesting angle for potential therapeutic translation. In the context of living donor transplantation, the donor alloantigen is known (and potentially available) for some time before the transplant is performed. In this setting, it may be possible to introduce the prospective transplant recipient to the donor alloantigen in advance of the transplant being performed and, through simultaneous administration of TGM (or a derivative), to 'reprogramme' the alloimmune response towards a less harmful one than would otherwise occur. Enhanced survival of experimental rat kidney allografts has been previously demonstrated when the transplants are performed after repeated administration of donor allogeneic splenocytes by oral gavage or injection into the prospective transplant recipient's portal vein[376]. Combination of this technique with TGM to further manipulate the alloimmune response might maximise the potential benefit for transplant recipients and is worthy of further investigation.

The data presenting here describing TGM is encouraging with regard to future therapeutic potential in a number of respects. First, TGM acts on both murine and human T cells to suppress proliferation and induce Foxp3. The level of Foxp3 expression induced by TGM is greater than that of TGF-β; a feature that has previously been shown to correlate with enhanced functional suppressive capacity of Treg[333]. Secondly, TGM is structurally completely distinct from TGF-β and is highly

unusual in having no shared homology with any other known molecules. This will likely enable evasion of the complex biological processes that regulate TGF-β signalling and may underlie the enhanced signalling downstream of the TGF-β receptor complex that has been observed. Thirdly, the main predicted adverse effect of TGF-β as a therapeutic agent is pathogenic fibrosis; whilst TGM is more potent in inducing Foxp3 expression that TGF-β, it is less potent in inducing fibrosis and therefore has the potential for a wider safe therapeutic dose range. Finally, TGM has been shown to be non-toxic at therapeutic dosages and to be functionally effective in prolonging allograft survival *in vivo*.

This project began with the hypothesis that *H. polygyrus* infection can protect transplanted allografts from immunological rejection. It is now clear infection can achieve this effect, but also that live infection is not required and at least one of the secreted products from *H. polygyrus* shows significant potential as a novel therapeutic candidate for further investigation.

7 References

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8 Appendix A – Interim Published Papers

OPEN

Helminths and Immunological Tolerance

Chris J.C. Johnston,[1,2] Henry J. McSorley,[1] Stephen M. Anderton,[2] Stephen J. Wigmore,[2] and Rick M. Maizels[1,3]

Current immunosuppression regimens for solid-organ transplantation have shown disappointing efficacy in the prevention of chronic allograft rejection and carry unacceptable risks including toxicity, neoplasia, and life-threatening infection. Achievement of immunological tolerance (long-term antigen unresponsiveness in an immunocompetent host) presents the exciting prospect of freedom from immunosuppression for transplant recipients. It is now 60 years since the first demonstration of immunological tolerance in animal models of transplantation, but translation into routine clinical practice remains elusive. Helminth parasites may provide novel strategies toward achieving this goal. Helminths are remarkably successful parasites: they currently infect more than one quarter of the world's population. It is now well established that the parasites' success is the result of active immunomodulation of their hosts' immune response. Although this primarily secures ongoing survival of the parasites, helminth-induced immunomodulation can also have a number of benefits for the host. Significant reductions in the prevalence of allergy and autoimmune conditions among helminth-infected populations are well recognized and there is now a significant body of evidence to suggest that harmful immune responses to alloantigens may be abrogated as well. Here, we review all existing studies of helminth infection and transplantation, explore the mechanisms involved, and discuss possible avenues for future translation to clinical practice.

Keywords: Allograft tolerance, Allograft rejection, Helminth.

(*Transplantation* 2014;97: 127–132)

Successful organ transplantation requires negotiation of a number of complex immunologic barriers. In addition to cell-mediated rejection resulting from mismatching of major and minor histocompatibility complexes (*1*), innate immune responses are invariably stimulated by a combination of surgery itself and organ damage resulting from ischemia and reperfusion (*2*). Ever-increasing demand for organ transplantation has unfortunately coincided with a steady decline in the availability of donor organs in recent decades (*3*). This has necessitated amended strategies to expand the donor pool that can result in donor organs of a lesser quality and/or greater degrees of immunologic mismatch (*3*).

Despite this, solid-organ transplantation has become the standard of care for numerous disease processes that result in organ failure. Advances in surgical techniques and immunosuppressive regimens mean that excellent short-term to medium-term graft survival has now come to be expected. However, recent scrutiny of long-term allograft survival data reveals that considerable improvements in the incidence and management of acute rejection have not been reflected in improved long-term outcomes (*4*). For living-donor kidney transplant recipients in the United States, organ graft survival half-life is almost unchanged (in 2005, this was 11.9 years compared with 11.4 years in 1989) (*5*). A similar disparity in short-term and long-term survival rates has also been seen in liver, lung, heart, intestine, and pancreas transplantation (*6*).

Current immunosuppression regimens used in solid-organ transplantation carry significant risks of toxicity, infection (*7*), and neoplasia (*8*). The incidence of neoplastic disorders such as posttransplantation lymphoproliferative disease, in particular, relates more to the intensity of immunosuppression than the specific agent used (*1*). All of these factors have fuelled the long-held ambition for allograft tolerance, defined as durable antigen-specific unresponsiveness in an immunocompetent host (*9*).

Despite the achievement of experimental murine allograft tolerance 60 years ago (*10*), translation to the clinical

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TABLE 1. Published studies reporting prolonged allograft survival in humans or laboratory animals in helminth-infected hosts, or in animals given helminth-derived products.

Authors	Parasite	Allograft model	Graft prolongation	*P*
Aboul-Enein et al. (*41*)	*Schistosoma mansoni*	Human skin	2.21	0.001
Hepiretihan et al. (*32*)	*Echinococcus multilocularis*	Rat heart	2.04	<0.05
Li et al. (*31*)	*E. multilocularis*	Rat liver	1.57	<0.05
Liwski et al. (*34*)	*Nippostrongylus brasiliensis*	Mouse heart	2.80	<0.03
Ledingham et al. (*33*)	*N. brasiliensis*	Rat kidney	3.30	<0.001
Araujo et al. (*42*)	*Nippostrongylus* NES	Rat kidney	2.22	<0.001
	S. mansoni	Mouse skin	1.5	<0.001
Svet-Moldavsky et al. (*43*)	*Trichinella spiralis*	Mouse skin	2.13	
Faubert and Tanner (*44*)	*T. spiralis*	Mouse skin	1.89	<0.001
Chimyshkyan et al. (*45*)	Infected mouse serum	Mouse skin	1.67	
	T. spiralis	Mouse skin	2.48	<0.001
Alkarmi et al. (*46*)	*Trichinella pseudospiralis*	Mouse skin	3.57	—
	T. pseudospiralis extract	Mouse skin	2.0	—
	T. spiralis	Mouse skin	3.57	—
	T. spiralis extract	Mouse skin	2.43	—

setting has been frustratingly slow. Recent developments in the understanding of regulatory cell populations have, however, allowed some ground for optimism (*11*). In particular, a recent trial of combining kidney transplantation with a simultaneous bone marrow transplant from single human leukocyte antigen (HLA)–mismatched donors has allowed for successful withdrawal of all immunosuppression in four of five patients (*12*). With this technique, patients developed transient mixed chimerism and lasting specific alloantigen unresponsiveness as a result. With the same technique, Scandling et al. have independently demonstrated similarly impressive outcomes with HLA-matched kidney transplantation: from a cohort of 16 patients, 8 have achieved rejection-free avoidance of immunosuppressive medication for more than 1 year, and a further 4 patients are in the process of withdrawal from medication (*13*). Although many would consider simultaneous bone marrow transplantation to present an unacceptable level of complexity and risk in the pursuit of solid-organ allograft tolerance (*9*), this important study has shown lasting intragraft regulatory cell populations and the successful allograft tolerance this can achieve in a clinical setting.

The ability of regulatory T cells (Treg) to mediate allograft tolerance in murine models is now well recognized (*14*). Expectations of successful translation of Treg therapy into the clinical setting have been high and preliminary clinical trials have now been completed in graft-versus-host disease (*15*) and hematopoietic stem cell transplantation (*16*) with modest but encouraging results. However, a number of obstacles and concerns persist. First, Good Manufacturing Practice-compliant ex vivo expansion of Tregs for subsequent reinfusion is a highly specialized process at a cost of approximately $40,000 per patient (*17*). Second, difficulty in identifying regulatory cell populations for purification and ensuring that they do not convert away from the regulatory phenotype presents the possibility of harm caused by inadvertent infusion of expanded effector T-cell populations (resulting in enhanced rejection or autoimmunity) (*18*). Finally, the specificity of Treg-induced immunosuppression is uncertain and may therefore present a risk of infection or neoplasia similar to that of current global immunosuppression regimens. Although results from early studies provide some degree of reassurance in this regard (*19*), analysis of long-term clinical safety data is still awaited.

Helminth Infections and Host Immunity

Helminth worms are particularly successful parasites; as recently as 1940, the prevalence of infection in children in some rural areas of the United States was as high as 70% (*20*) and the current rate of chronic infection stands at more than one quarter of the world's population (*21*). Helminths' success is now recognized to be the result of active modulation of their hosts' immune response (*21*). In addition to facilitating chronic infection, parasite-derived dampening of the host systemic immune response also results in reduced reactivity to unrelated "bystander" allergens and autoantigens or alloantigens. In many cases, this effect is of some considerable benefit to the host. Recent studies have shown abrogation of multiple disease processes in the presence of helminth infection, including allergic airway inflammation, encephalitis, inflammatory bowel disease, rheumatoid arthritis, and type I diabetes in experimental models (*22*). Epidemiologically, it is well established that the prevalence of allergic and autoimmune disorders is higher in developed countries compared with developing nations. Recent cohort studies investigating allergic responses in children demonstrated reduced skin responses to antigen testing in helminth-infected subjects compared with controls and found that this effect was eliminated after clearance of infections with antihelminthic therapy (*22*). Similarly, patients with multiple sclerosis in Argentina who adventitiously acquired helminth infections were found to stay in remission but relapsed after antihelminthic drug treatment (*23*). A recent phase I trial of experimental helminth infection with *Trichuris suis* as a therapy for Crohn's disease revealed no adverse effects (in 29 patients) and showed promising considerable reductions in disease severity scores (*24*). Large-scale Good Manufacturing Practice–compliant production of *T. suis* for clinical use has

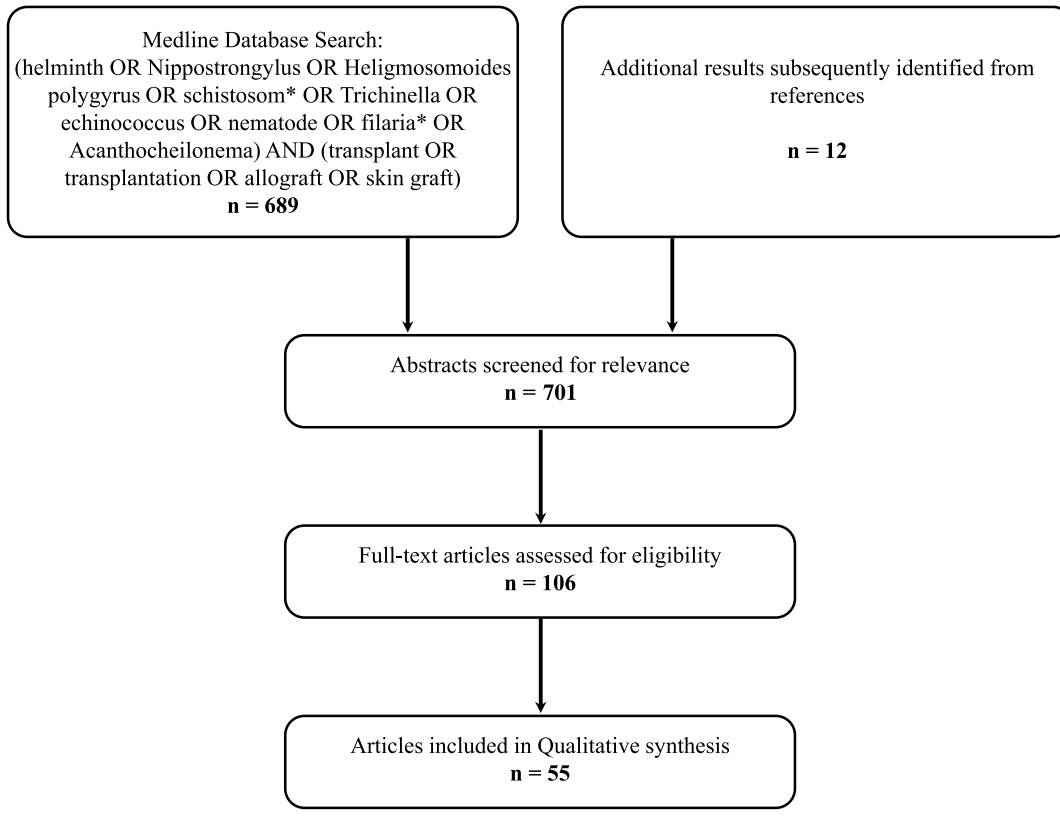

FIGURE 1. Search strategy.

been approved by the U.S. Food and Drug Administration and a number of multicenter randomized controlled trials have commenced (*25*). In the absence of a corresponding analysis of allograft survival in helminth infected hosts, we performed a publication search (Fig. 1) and report here a synthesis of the relevant literature.

Echinococcus Tapeworms

The metacestode *Echinococcus multilocularis* (*Em*) is endemic among foxes in many parts of Europe and China such that humans can be affected as accidental hosts. Infection can result in severe disease with a clinical course resembling that of a malignant primary liver tumor (hepatocellular carcinoma) (*26*). Radical surgical resection of liver lesions has been shown to be effective in improving survival and orthotopic liver transplantation is now largely accepted as appropriate treatment for advanced disease (*27*). Disease progression caused by the parasite has been found to advance rapidly in the presence of host immunosuppression either as a result of medication (*1, 28*) or HIV infection (*2, 29*). This finding led to guidelines recommending reduced immunosuppression regimens after liver transplantation for *Em* (*30*) and long-term follow-up reported unexpectedly satisfactory tolerance of the allografts (*3, 27*). Tao et al. have subsequently corroborated this finding in an experimental rat model of liver transplantation with *Em* infection (*3, 31*) (Table 1). In this study, survival after orthotopic liver transplantation found to be significantly prolonged for *Em*-infected rats compared with naïve controls (15.5±3.9 vs. 9.9±2.3 days; $P<0.05$). The *Em*-infected group was also found to have reduced $CD4^+$, $CD8^+$, and $CD28^+$ T-cell populations in peripheral blood, raised serum interleukin (IL)-10 levels, and reduced histologic liver allograft rejection scores, all of which reached statistical significance ($P<0.05$) (*4, 31*). More recently, Hepiretihan et al. have shown a that *Em* infection exerts a similar protective effect against rejection of rat heart allografts (16.2±3.2 vs. 7.9±1.9 days) (*5, 32*). This was associated with a reduction in graft-infiltrating $CD8^+$ lymphocytes and a shift toward a Th2 cytokine profile in the serum of peripheral blood. In the clinical setting, eradication of *Em* infection usually proves impossible. It therefore remains as yet unclear as to whether graft protection is afforded by an ongoing influence of the parasite or as the result of a Th2 cytokine environment at the time of alloantigen presentation.

Nippostrongylus Roundworms

Beneficial enhancement of allograft tolerance in the presence of helminth infection has now been demonstrated with a number of parasite species and does not seem to be restricted to specific organs or host species (Table 1). In 1996, Ledingham et al. demonstrated marked improvement in the survival of kidney allografts in rats infected with the gastrointestinal nematode, *Nippostrongylus brasiliensis* (*Nb*), or inoculated with its secretory products compared with naïve controls (32±10, 21±4.6, and 9.7±1.2 days, respectively; $P<0.001$) (*6, 33*). Representative histologic examination 5 days after transplantation showed a dramatic reduction of graft cellular infiltration in the *Nb*-infected group and this finding was supported quantitatively with flow cytometric analysis of digested allograft single-cell suspensions (84% and

81% reduction of $CD8^+$ and $CD4^+$ lymphocytes, respectively). Although the graft protection afforded by *Nippostrongylus* excretory-secretory products (NES) was less pronounced, the pharmacokinetic profile of the active mediator(s) in NES is unknown and this may therefore be a purely dose-dependent difference.

The same group later showed similar (2.8-fold) graft protection in a mouse cardiac allograft model (*7, 34*). *Nb* infection is known to induce a strong Th2 response in its host (*8, 35*), leading those authors to hypothesize that polarization away from Th1-mediated allograft rejection may afford allograft protection. *Nb* usually achieves only a limited infection in rodents—most mouse strains can clear the infection within 10 days of inoculation with third-stage larvae (*1, 35*). The finding that mouse heart allografts can survive for considerably longer than the period of infection (*9, 34*), presents the exciting therapeutic prospect that graft protection is afforded by T-cell "phenotype switching" at the time of alloantigen presentation rather than a mechanism dependent on persisting parasite infection. Enzyme-linked immunosorbent assay analysis of mixed lymphocyte reactions supports this hypothesis in demonstrating a Th2 cytokine profile (IL-4 and IL-6) in alloreactive lymphocytes from *Nb*-infected mice compared with naïve controls (*34, 36*).

Schistosome Flukes

Schistosoma is a genus of blood-borne trematode with a current prevalence of infection estimated at more than 200 million people worldwide (*10, 37*). In light of the very widespread prevalence of schistosomiasis and the diminishing supply of suitable cadaveric donor organs for transplantation, a number of human liver (*11, 38, 39*) and kidney (*12, 40*) transplants in patients with clinical schistosomiasis have been performed (donor and recipient, donor alone, and recipient alone). No attempts at reducing immunosuppression or analyzing differences in rejection rates have as yet been reported. However, one remarkable study has looked at the differential rejection of full-thickness skin grafts in Egyptian patients with established schistosomiasis compared with healthy volunteers. Aboul-Enein et al. (*9, 41*) recruited 19 patients with advanced *Schistosoma mansoni* infection and 16 parasite-free volunteer controls. Then, 2.5-cm-diameter full-thickness skin grafts were applied to the volar forearm. Two grafts were performed for each patient: one ABO-matched allograft from a noninfected donor and one autograft control. Grafts were assessed daily for signs for rejection and rejection was then confirmed histologically. The control group rejected their allografts after a mean of 10.06±3.21 days. Of the *Schistosoma*-infected patients, in 16 cases, rejection occurred after a mean of 22.25±6.46 days. The remaining three infected patients showed no signs of rejection 60 days after the grafting procedure. Notably, the HLA status of donors and recipients was unknown in this study; therefore, the three cases of long-term graft tolerance may well be the result of coincidental HLA matching. In spite of this significant caveat, the difference in rejection times between the two groups was highly significant ($P<0.001$) and therefore unlikely to be the result of differences in HLA matching alone.

Allograft protection with *Schistosoma* infection has previously been shown in a murine experimental model. In 1977, Araujo et al. found a highly significant difference in the rejection of fully allogeneic skin grafts in *S. mansoni*–infected versus naïve recipient mice (*14, 42*). No difference was found after 30 days of infection, but for grafts performed after 60 days of infection, infected recipients tolerated their grafts for an average of 50% longer than naïve controls. A strongly positive correlation between graft survival and the number of live parasites remaining in the recipient was also seen (r=0.096).

Trichinella

Finally, murine experimental models of other helminth species have also demonstrated enhanced tolerance of skin allografts. *Trichinella spiralis* is a small nematode that encysts in mammalian muscle and can affect humans who consume infected meat. Suppression of skin allograft rejection in mice infested with *Trichinella* was first described by Svet-Moldavsky et al. in 1969 (*15, 43*) and subsequently confirmed by Faubert and Tanner (*16, 44*) and Chimyshkyan et al. (*18, 45*). In 1995, Alkarmi et al. performed fully allogeneic skin grafts (C57BL/6 to BALB/c recipients and vice versa) on multiple groups of mice at varying time points after infection (*17, 46*). Graft protection was found to be critically dependent on the timing of skin transplantation in relation to initial infection and a maximum effect of 3.5-fold prolongation of graft survival was found when the transplants were performed 3 days after initial infection. Repeated intraperitoneal injection of parasite secretions (culture supernatants) replicated the effect of active infection in a dose-dependent fashion with an observed maximum twofold prolongation in graft survival (*18, 46*).

Evolution of Regulation

Coevolution of helminths and humans over millions of years (*19, 47*) has resulted in multiple effective mechanisms of immunomodulation, which may individually or in combination be responsible for the prolongation of allograft survival. Certainly, it is now clear that helminths act via multiple distinct and synergistic pathways to down-regulate host immunity. Expansion of Treg populations in response to helminth infections such as *Heligmosomoides polygyrus* (*20, 48*) and *S. mansoni* (*21, 49*) is one well-recognized mechanism, but the same parasites can also engender immunosuppressive activity in B-cell populations as well as modified dendritic cell and macrophage populations (*21, 22*).

In this context, it is possible that therapeutic extension of graft survival would also require more than one particular immunomodulatory pathway. With respect to Treg expansion, exogenous IL-2:anti-IL-2 antibody complex is a potent short-term stimulant of Treg populations, which can effect long-term tolerance of allogeneic islet grafts in the absence of immunosuppression (*22, 50*). However, multiple attempts to achieve similar tolerance of allogeneic skin grafts (BALB/c to C57BL/6) have failed (*23, 51*). It is well known in the experimental and clinical setting that tolerance of skin allografts presents a particular challenge (compared with the solid-organ transplants of heart, liver, or kidney). Important factors to overcome are likely to be the large proportion of resident dendritic cells in skin (*24, 51*) and more potent Toll-like receptor stimulation by colonizing microbes (*25, 52*). Failure of IL-2:anti-IL-2 complexes to achieve the same level of protection of fully allogeneic skin grafts against rejection (*26, 51*) that is seen in *Schistosoma* infection (*27, 41*) strongly

suggests that Treg-independent mechanisms also play a critical role in helminth-derived allograft protection.

CONCLUSIONS

Enhanced allograft tolerance with helminth infection has now been demonstrated in multiple species across multiple organ allograft models (mouse heart and skin; rat heart, liver, and kidney). These experimental data are consistent with historical results of skin grafting in established human schistosomiasis and supported by more recent anecdotal suggestions of reduced immunosuppression requirement after liver transplantation for human *Echinococcus* infection (*27*). Thus, the possibility can now be entertained of including specific live (nonpathogenic) helminth infection, or defined products from immunoregulatory helminths, in future transplantation protocols. Ongoing trials of live *T. suis* therapy in inflammatory bowel disease (*25*) are keenly awaited as potential pathfinding studies for translation of this concept to the clinic.

There are indeed multiple potential opportunities for helminthic and helminth product therapy in transplantation. The most promising is with living-donor transplantation, whereupon a course of helminthic therapy may be commenced before the time of transplantation, allowing alloantigen presentation to occur in a tolerogenic environment (either at the time of the transplantation itself or with known defined alloantigens beforehand). Although treatment with active helminth infection has been shown to be a safe therapeutic approach (*25*), reports of mild gastrointestinal side effects do exist and might limit patient acceptability (*53*). Identification and synthetic production of the active compounds within helminthic secretions for novel pharmaceutical intervention is a definitive goal and the focus of much attention (*54*). Measured against current transplant immunosuppression regimens with multiple serious adverse effects and inadequate long-term organ protection against rejection, therapy with helminths or their products presents the exciting opportunity of a safe, effective, and long-overdue alternative.

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Video Article

Cultivation of *Heligmosomoides Polygyrus:* An Immunomodulatory Nematode Parasite and its Secreted Products

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Abstract

Heligmosomoides polygyrus (formerly known as *Nematospiroides dubius,* and also referred to by some as *H. bakeri*) is a gastrointestinal helminth that employs multiple immunomodulatory mechanisms to establish chronic infection in mice and closely resembles prevalent human helminth infections. *H. polygyrus* has been studied extensively in the field of helminth-derived immune regulation and has been found to potently suppress experimental models of allergy and autoimmunity (both with active infection and isolated secreted products). The protocol described in this paper outlines management of the *H. polygyrus* life cycle for consistent production of L3 larvae, recovery of adult parasites, and collection of their excretory-secretory products (HES).

Video Link

The video component of this article can be found at http://www.jove.com/video/52412/

Introduction

Heligmosomoides polygyrus is a natural murine gastrointestinal helminth that is closely related to highly-prevalent human nematode parasites [1]. In contrast to other nematode models such as *Nippostrongylus brasiliensis*, *H. polygyrus* consistently establishes chronic infection in mice as a direct result of multiple powerful immunomodulatory mechanisms it employs to suppress the host immune response [2].

H. polygyrus has a direct life cycle: infective L3 larvae are ingested by feco-oral transmission (or administered by oral gavage in the laboratory setting), whereupon they migrate to the subserosal layer of the duodenum and encyst before returning to the intestinal lumen as adult worms approximately eight days after initial infection. Mating and egg production occurs by day 10 and it is possible to harvest adult worms for culture and collection of excretory-secretory products from day 14 onwards [3]. *H. polygyrus* also interacts with the commensal microflora, with increased Lactobacilli present in infected susceptible mice [4-6], and increased levels of *H. polygyrus* infection following exposure of mice to Lactobacilli [6].

Active infection with *H. polygyrus* has been shown to protect against immunopathology in many animal models of autoimmunity [7-10], colitis [11,12] and allergy [13-16]. There has consequently been great interest in the potential of Excretory-Secretory molecules from this parasite ("HES") to down-modulate pathology in vivo [17,18]. Indeed, protective effects are seen following treatment of mice with HES products [19] through pathways which are now being identified [18,20]. Here we describe a protocol for the reliable production of *Heligmosomoides polygyrus* and recovery of its secreted products that can be further utilized for a range of functional biochemical and immunological investigations.

Protocol

NOTE: All procedures in this protocol are performed in accordance with guidelines set out by the United Kingdom Home Office and the University of Edinburgh Veterinary Services.

1. Infection of Mice by Gavage

1. Store *Heligmosomoides polygyrus* L3 larvae in distilled water for up to six months at 4 °C.
2. Before use, wash L3 larvae three times in distilled water: centrifuge at 300 x g for 10 min (with brake), remove all but 500 µl of water (to avoid disturbing pelleted L3 larvae) and resuspend the pellet each time.

3. For the third wash, add water to an exact volume (typically 40 ml) and aspirate 20 µl with a 200 µl tip cut to widen its aperture. Place two 20 µl samples on the surface of a 60 mm culture dish and count the L3 larvae (usually mobile, and best viewed under 50X magnification with a dissecting microscope). Resuspend the final pellet in distilled water to a concentration of 2,000 L3 larvae per ml.
4. For life cycle production, infect 8 week-old F1 (C57BL/6xCBA) mice with 400 *H. polygyrus* L3 larvae in 200 µl of distilled water by oral gavage (restrain mice in the upright position by the scruff of the neck and gently pass the blunt gavage needle through the mouth and esophagus into the stomach). Agitate thoroughly prior to each infection (larvae settle quickly in water) and aspirate 200 µl in a 1 ml syringe; use a dedicated gavage needle with a rounded end. For experimental infection of younger mice (6-8 weeks old), or other inbred strains (*e.g.* C57BL/6 or BALBc), infect mice with 200 L3 larvae.

2. Propagation and Maintenance of *H. polygyrus*

1. Place charcoal in the center of a large plastic tub and allow cold tap water to run over it for a minimum of 30 min (unwashed activated charcoal is toxic to L3 larvae). Drain the water from the tub and place the charcoal on two layers of absorbent paper, leaving it exposed to room air until completely dry.
2. If eggs are required, scrape feces first out of the colon with forceps (and scissors if necessary). If a large number of L3 larvae is needed, place mice on a wire grid and collect fecal pellets over several days.
3. Mix the feces with granulated charcoal at a ratio of at least a 1:1, to achieve a consistency just damp enough to adhere to filter paper. Smear a thin layer on the center of some dampened filter paper in a petri dish and place this in a humid box (add some damp paper towel and a dish of water) in the dark for 12-14 days.
4. Remove L3 larvae from day 7 onwards, and collect them on at least two occasions before the paper is discarded. The larvae form a ring around the edge of the filter paper; lift the filter paper out of the petri dish and rinse the larvae that are left of the plate (using a pipette and 5 ml of sterile water per plate) into a 50 ml tube.
5. Lift off the filter paper and harvest the remaining larvae left on the plate with distilled water into a 50 ml tube. Centrifuge the effluent solution at 300 x g for 10 min. Wash the larvae three times with distilled water and then store at 4 °C in up to 50 ml of distilled water until required.
   NOTE: Larvae remain viable and infective for at least 6 months.

3. Collection of Adult *H. polygyrus* Worms

1. Prepare the modified Baermann Apparatus in advance as shown in **Figure 1**.
2. Cull mice fourteen days after infection.
3. Wash the abdomen with 70% ethanol. Cut the skin over the abdomen and pull back to reveal the anterior abdominal wall. Make midline incision to enter the peritoneal cavity.
4. Remove the entire small and large intestine (from proximal duodenum to distal rectum). Place into a dry Petri dish.
5. Straighten the gut along its entire length; excise the feces-containing colon; place this into a separate dish for egg preparations later.
6. Excise the proximal 20 cm of small intestine that contains the adult worms –identified by the relatively thick wall of the duodenum and often a red appearance due to the intra-luminal worms. Place into a (100 mm diameter) Petri dish with 5 ml of Hanks' Solution, warmed to 37 °C (two specimens per dish).
7. Open the worm-filled proximal gut portion longitudinally with scissors (round-ended scissors are best for this), and scrape down inside of gut lining with two glass slides to remove the worms. Then discard the clean gut wall.
8. Tip worms into small muslin bags, staple closed and secure with paperclips around the edge of the glass funnel (**Figure 1**).
9. Fill funnel with Hanks' Solution and add approximately 4 Petri dishes of worms into each funnel.
10. Place apparatus in 37 °C incubator for 1-2 hr, gently agitating half way through to dislodge debris from the gut preparation that may occlude the muslin filter. Take care to avoid spillage of debris outside the muslin bag – this will cause contamination of the final HES preparation. Adult worms should have slowly migrated through the muslin cloth and settled at the bottom of the glass test tube. Carefully detach the test tube from the connecting rubber hose over the sink (taking care to avoid losing worms at this point).
11. Using a plastic pipette, transfer the worms into a 50 ml tube and wash six times with Hanks' Solution (allow worms to settle with gravity, remove media with a stripette, add 40 ml of Hanks' Solution and repeat five times).
    NOTE: worm culture must be kept sterile from this point onwards.
    1. Move to a laminar flow hood and wash another six times in sterile Hanks' Solution supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin, ready for *in vitro* culture.
12. Count the adult worms recovered by taking two samples of 20 µl taken up with a yellow tip cut to widen its aperture; expect approximately 50% of the quantity of inoculated larvae.

4. Setting Up Cultures for HES: Medium Preparation, Washing Adult Worms

1. Soak the worms from 3.11 in approximately 10 ml of RPMI supplemented with 10% Gentamycin for 20 min, leaving the tube resting at an angle to ensure worms are fully covered.
2. Perform this in a laminar flow hood: wash again six times with Hanks' Solution (supplemented with 5 U/ml penicillin and 5 µg/ml streptomycin).
3. Prepare *H. polygrus* media.
4. Maintaining sterility in a laminar flow hood, to 500 ml of RPMI1640, add 11.1 ml of 45% glucose (final concentration 1.2% as RPMI1640 contains 0.2% glucose), 5 ml of 100x PenicIllin-Streptomycin (final concentration 5 U/ml penicillin, 5 µg/ml streptomycin), 5 ml of L-glutamine (final concentration 2 mM), and 5 ml of Gentamycin (final concentration 1%). Do not add FCS.
5. Aliquot worms into vented T25 flasks, approx. 1,000 worms in 15 ml *H. polygyrus* media per flask, and place upright in 37 °C incubator (5% CO_2) for 3 weeks.

5. Preparation of HES

1. Collect HES-containing culture media from cultures at intervals of no longer than twice per week, setting aside the first collection after 24 hr of culture (due to high levels of LPS contamination and host proteins – can be processed separately or discarded). Keep each collection separate and clearly labeled with the date and batch number. Replace with an equal volume of *H. polygyrus* media on each occasion.
2. Centrifuge HES-containing media at 400 x *g* for 5 min. Then filter sterilize through 0.2 µm low-protein-binding filters into 50 ml tubes. Store in the -20 °C freezer clearly labeled with date of worm harvest and date of HES collection.
3. After 21 days of HES collection from culture, discard worms.
4. Pool 500-1,000 ml of HES supernatant (usually from frozen stock, and not including the first 24 hr collection) and concentrate over a 3,000 MWCO filter in the ultrafiltration device under nitrogen pressure.
   NOTE: Be very careful not to let the filter run dry.
   1. To set up the filter device, first wash the 3 kDa membrane shiny side down in a 1 liter beaker with distilled water for 3 x 20 min whilst stirring. Assemble the ultrafiltration device as per manufacturer's instructions with filter membrane shiny side up. Place in cabinet at 4 °C and pass 50 ml of distilled water through before starting to concentrate pooled HES.
   2. Add each tube of HES into the filtration device as required (100-140 ml per day), until the volume is concentrated down to 2-5 ml.
5. In order to remove contaminants from the HES-containing culture media, add 50 ml of pyrogen-free PBS to the filtration device and and then concentrate down to approximately 2 ml. Repeat this step twice (150 ml of PBS in total). Transfer HES into a 15 ml tube, filter sterilize (with a 0.2 µm filter) in a laminar flow hood and measure protein concentration using a spectrophotometer (E_{280} = 10) or by Bradford assay.
6. Aliquot, label with batch number and date, and freeze at -80 °C.
7. Perform a chromogenic LAL assay according to the manufacturer's protocols on each batch of HES prior to use. If LPS levels are greater than 1 U LPS per 1 µg protein, consider not using this batch for *in vivo* experiments or *in vitro* cultures.
8. Process the HES collected at 24 hr separately in the same manner; it will contain LPS and some host proteins and, while not suitable for functional experiments, it is a useful source of individual molecules that may be isolated by monoclonal antibody affinity purification.

Representative Results

Susceptibility to infection with *H. polygyrus* is controlled to a large degree by the genetic background of the mouse strain (**Table 1**); C57BL/6 and CBA mice are highly susceptible [21,22]. For maintenance of the parasite life cycle, the F1 hybrid between these two strains has been chosen for its ability to withstand much higher worm burdens without morbidity (excessive intestinal epithelial damage) compared to either parental strain. Oral gavage of 400 L3 larvae is used to maintain the life cycle in F1 mice (resulting in adult worm burdens shown in **Figure 2**), whilst a dose of 200 L3 larvae is generally used for experiments in homozygous inbred strains (*e.g.*, C57BL/6 or BALBc). However, this dosage may need to be reduced depending on environmental co-factors that may differ between animal facilities, such as variations in gut flora.

Batches of HES have proven reproducible efficacy in functional assays and in protein composition; moreover, when supernatants from each successive week of culture were analysed, up to a total of 4 weeks, the protein profiles were found to be very similar (**Figure 3**)**.** When HES concentration is measured by Bradford assay (see **5.5**), the total protein is usually approximately 1 mg/ml (**Figure 4**).

An alternative method of concentrating HES is with a centrifugal concentrator (eg Vivaspin 3-kDa cut-off membrane), in which samples are spun at up to 4,000 g in a swing-out rotor centrifuge, removing buffer salts and low molecular weight components. Centrifugal concentration is best suited to small processing volumes (1-10 ml) and are limited to a maximum concentration factor of approximately 30x.

When collecting HES, avoidance of contamination is critical. To avoid contamination with host molecules, we discard HES-containing culture media from the first 24 hr after adult worm harvest from the mouse intestine. We also quantify the level of LPS contamination in each batch of HES with a Chromogenic LAL assay (see 5.7). 1 U of LPS equates to ~100 pg LPS and levels below this are considered negligible [23]. In our hands, most batches of HES are significantly below this limit, the mean concentration of LPS in HES being 0.23 U/µg (**Figure 5**). The effects of LPS in *in vivo* models of pathology (for instance the suppression of asthmatic responses) requires at least 10 ng of LPS [23]. Hence *in vivo* administration of 5 µg HES from a batch with 100 pg LPS/µg HES will include 500 pg LPS, well below the effective concentration where LPS becomes a problem.

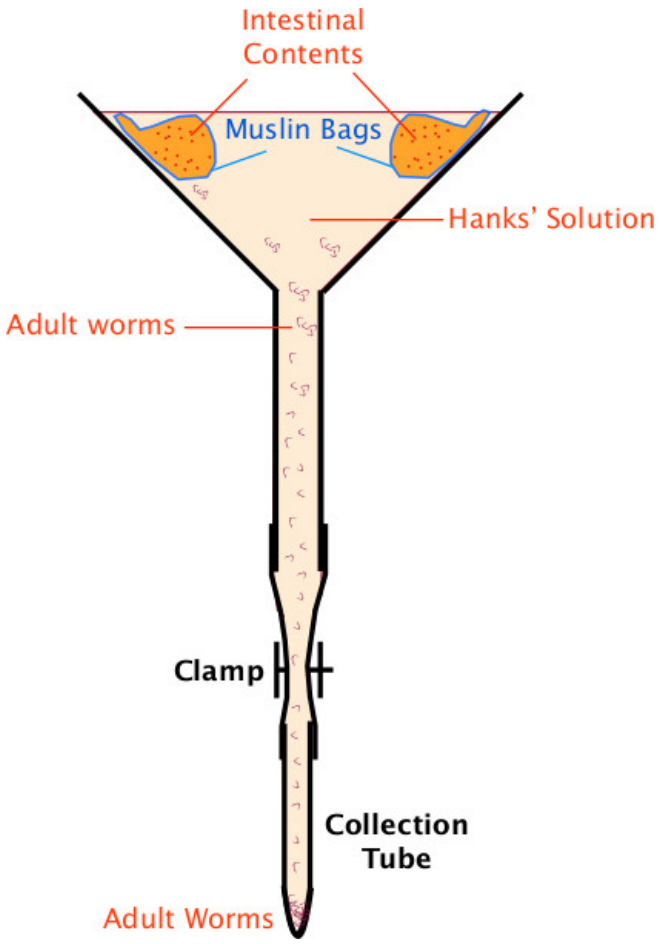

Figure 1: Baermann Apparatus Baermann Apparatus setup for collection of adult *H. polygyrus* (as described in section 2).

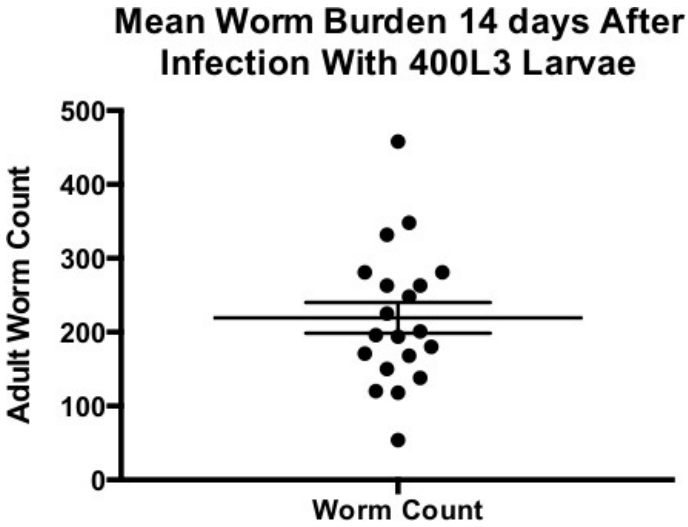

Figure 2: Mean Worm Burden 14 Days After Infection with 400 L3 Larvae Mean worm burdens 14 days after infection with 400 L3 larvae. Data points shown are from 19 separate rounds of infection of C57BL/6xCBA mice. Mean ± SEM shown.

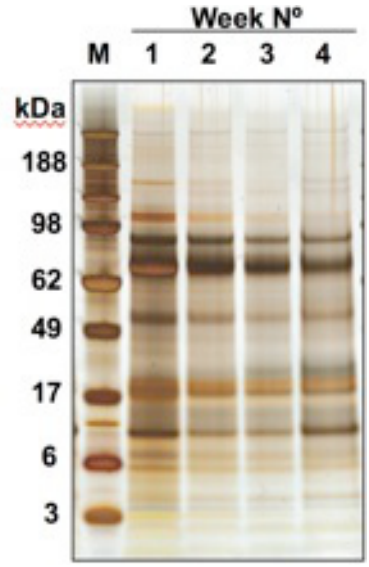

Figure 3: Protein Profiles of HES From Successive Weeks in Culture SDS-PAGE profile of HES proteins collected in successive weeks of culture.

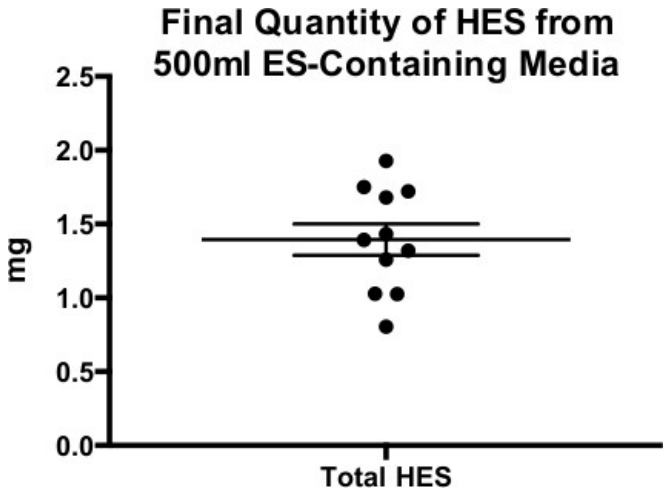

Figure 4: Final Quantity of HES from 500 ml ES-Containing Media Yield of HES protein from 11 different batches derived from approximately 500 ml of culture supernatant. Mean ± SEM shown.

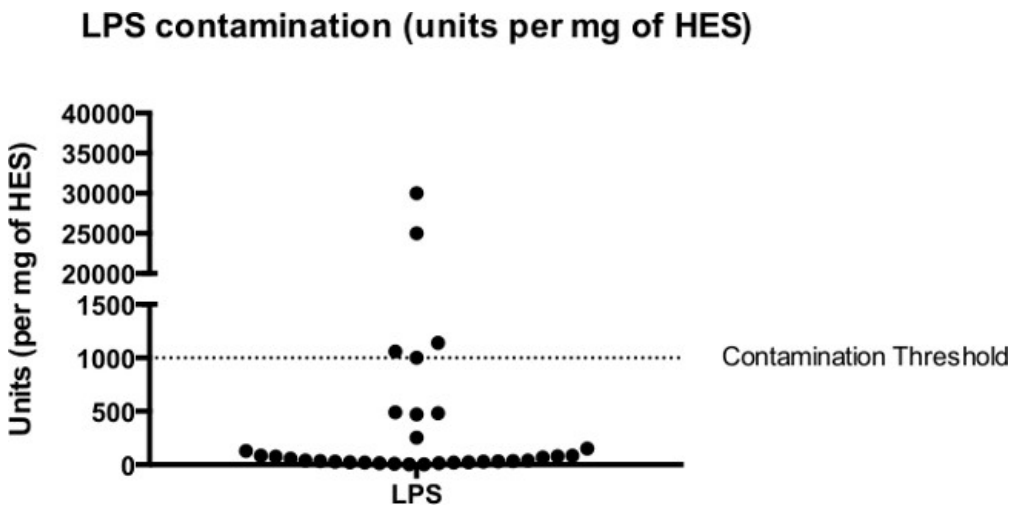

Figure 5: LPS Contamination of HES Levels of LPS contamination in 41 batches of HES measured by the Limulus ameobocyte assay. Median LPS concentration = 86 U per mg of HES.

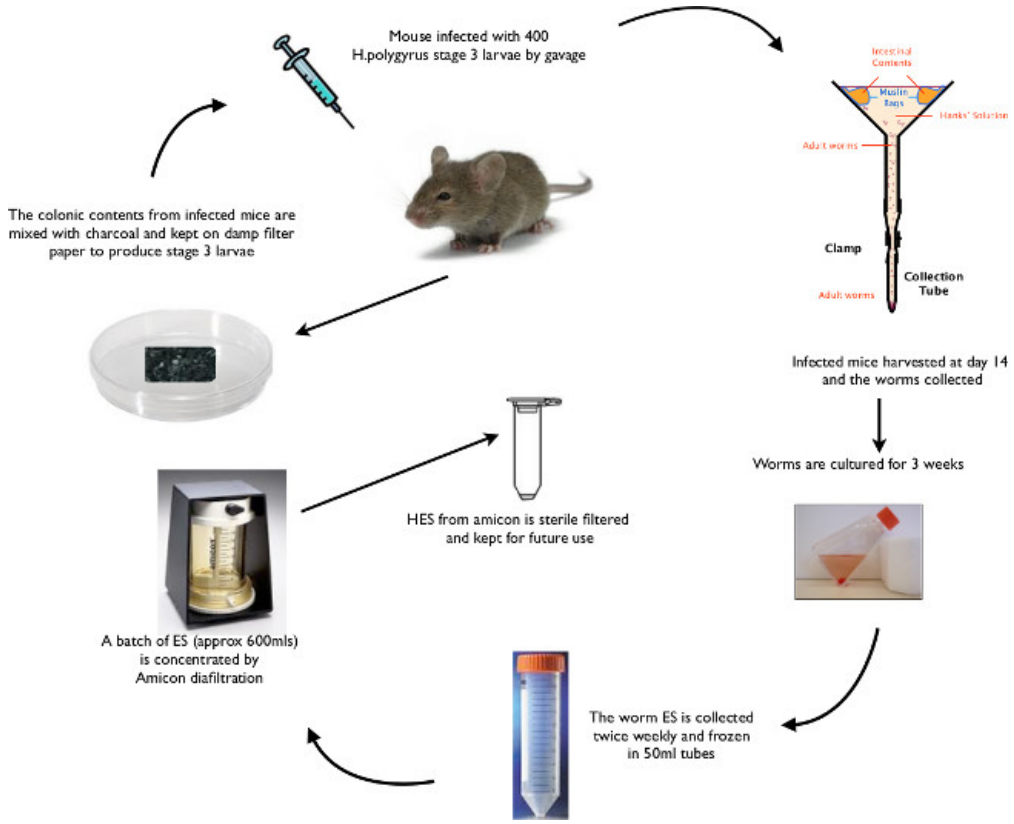

Figure 6: Animated Schematic of *H. polygyrus* Life Cycle Summary of key life cycle stages from oral gavage of L3 larvae, through recovery of larvae and adult worms to isolation of HES.

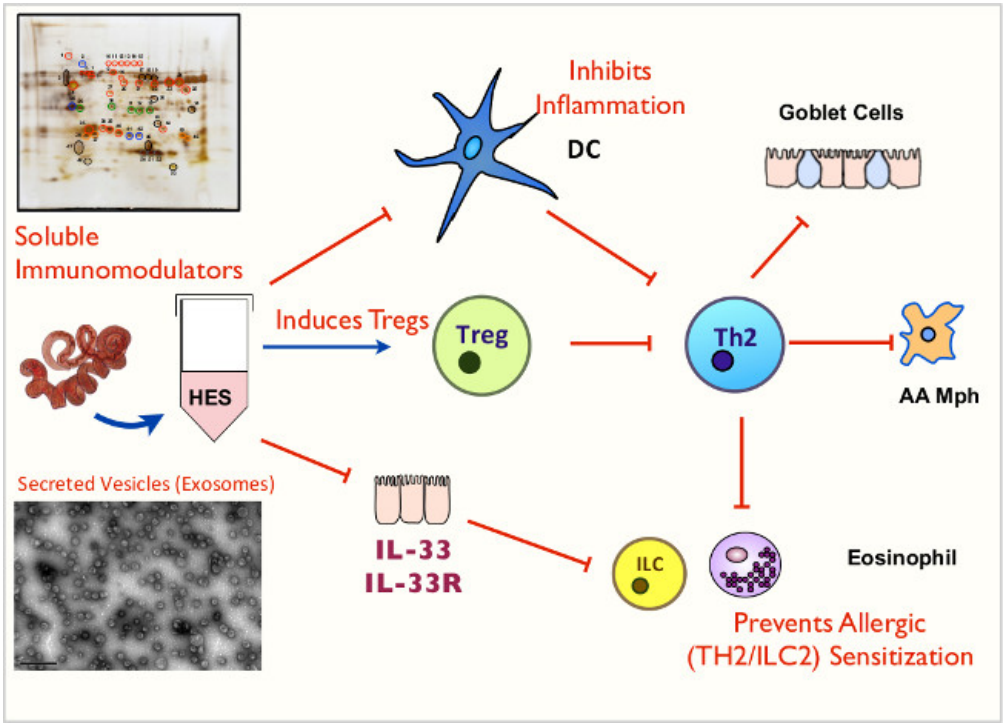

Figure 7: Animated Schematic of HES and its Functions Key immunomodulatory effects of soluble mediators and exosomes contained within HES.

Genotype (and background strain)	Primary Infection Phenotype	Reference
Inbred strains		
A/J, CBA, C3H	Highly susceptible	22,29
C57BL/6 and C57BL/10	Susceptible	22,30
BALB/c, DBA/2, 129/J	Intermediate	22,31
NIH, SJL, SWR	Low susceptibility	22,32
Transgenic for cytokines or cytokine receptors		
IL-1β$^{-/-}$	More susceptible	33
IL-1R-/-	Less susceptible (a); no change in susceptibility but increased granulomas (b)	(a) [33]
		(b) [34]
IL-2Rβ Transgenic (C57BL/6)	Resistant	35
IL-4-/-	Higher fecundity	36
IL-4R-/- (C57BL/6 or BALB/c)	Highly susceptible	22
IL-6$^{-/-}$ (BALB/c or C57BL/6)	Highly Resistant	37
IL-9 Transgenic (FVB)	Resistant	38
IL-21R $^{-/-}$ (C57BL/6)	Deficient Th2, decreased granuloma gormation	39,40
IL-25-/- (BALB/c)	More susceptible	33
IL-33R (T1/ST2) -/- (BALB/c)	No change in susceptibility	33
TGFβRIIdn (C57BL/6)	High Th1, Increased susceptibility	41,42
Transgenic for T cell markers		
CD28–/– (BALB/c)	Marginally higher fecundity	43
CD86 (B7-2)–/– (BALB/c)	Higher fecundity	44
OX40L–/– (BALB/c)	Higher fecundity	45
Transgenic for innate Immune loci		
Type 1 interferon receptor (IFNAR)-/- (C57BL/6)	Higher fecundity, more granulomas	34
MyD88–/– (C57BL/6)	More resistant, more granulomas	34
C-KitW/Wv (WBB6)	More susceptible	46,47

Table 1: Primary infections with *H. polygyrus* in genetically different and gene-targetted mouse strains.

Discussion

The life cycle of *H. polygyrus* proceeds in a reliably consistent fashion. Following natural ingestion or oral gavage of L3 larvae on day 0, cysts begin to form under the duodenal serosa by day 5, progressing to larval moults and then emerging as adult worms into the gut lumen from day 10, eggs can be seen in feces from day 14 and granulomas are visible on the duodenal serosal surface from day 21. The protocol described above (and summarized in **Figure 6**) allows for high-throughput production of *H. polygyrus* excretory-secretory products (HES), in addition to reliable recovery of viable L3 larvae for future experimental and life cycle infections.

H. polygyrus infection has been shown to be protective in models of asthma dependent on OVA or Der p 1 (House dust mite allergen)[14]. Furthermore, suppression of airway inflammation could be transferred with $CD4^+CD25^+$ regulatory T cells [14] or $CD19^+CD23^{hi}$ regulatory B cells [24] from non-sensitised, *H. polygyrus*-infected mice. In models of autoimmunity, *H. polygyrus* infection has been shown to be suppressive in the experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis [9], and suppression can be transferred with either CD4+ T cells or CD19+ B cells from infected mice [24].

Heligmosomoides polygyrus excretory-secretory products (HES) modulate the host immune response and suppress Th2-mediated inflammation by a number of mechanisms (outlined in **Figure 7**), including: a) Inhibition of dendritic cell responsiveness to stimulation [25], b) induction of $CD4^+Foxp3^+$ regulatory T cells [18]. and c) blockade of IL-33 production [20]. HES has been shown to be protective when administered at sensitization or challenge in the OVA-alum model of asthma [19] and also when administered intranasally with Alternaria extract allergen [20], through suppression of early IL-33 release. Avoidance of LPS contamination of HES is often crucial for the success of future immunological

experiments. In the protocol outlined here, the critical steps in achieving this are to ensure that debris contained within the muslin bags of the Baermann Apparatus does not enter the final HES preparation (see 2.10) and to set aside ES solution from the first 24 hr of culture (see 5.1).

Over recent years, HES has been thoroughly characterized at the proteomic level with over 370 distinct proteins identified [26,27]. In addition, ES from the 4th stage larvae has been subjected to proteomic analysis [28]. Ongoing work includes characterizing the major glycan components of HES, known to be strongly immunogenic [3], and secreted micro-RNAs that are encapsulated in 50-100 nm vesicles or exosomes (Buck *et al.*, submitted for publication). With the establishment of reproducible protocols for the collection of ES from this highly immunomodulatory parasite, and with extensive proteomic data identifying the molecular components of HES, the stage is now set for the identification and therapeutic testing of individual molecules from *H. polygyrus* that can mediate key effects on the host immune system.

Disclosures

The authors have no conflicts of interest.

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Research paper

TGF-β in tolerance, development and regulation of immunity

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ABSTRACT

The TGF-β superfamily is an ancient metazoan protein class which cuts across cell and tissue differentiation, developmental biology and immunology. Its many members are regulated at multiple levels from intricate control of gene transcription, post-translational processing and activation, and signaling through overlapping receptor structures and downstream intracellular messengers. We have been interested in TGF-β homologues firstly as key players in the induction of immunological tolerance, the topic so closely associated with Ray Owen. Secondly, our interests in how parasites may manipulate the immune system of their host has also brought us to study the TGF-β pathway in infections with longlived, essentially tolerogenic, helminth parasites. Finally, within the spectrum of mammalian TGF-β proteins is an exquisitely tightly-regulated gene, anti-Müllerian hormone (AMH), whose role in sex determination underpins the phenotype of freemartin calves that formed the focus of Ray's seminal work on immunological tolerance.

1. Introduction

The TGF-β superfamily is an ancient metazoan protein class which cuts across cell and tissue differentiation, developmental biology and immunology. Its many members are regulated at multiple levels from intricate control of gene transcription, post-translational processing and activation, and signaling through overlapping receptor structures and downstream intracellular messengers. We have been interested in TGF-β homologues firstly as key players in the induction of immunological tolerance, the topic so closely associated with Ray Owen. Secondly, our interests in how parasites may manipulate the immune system of their host has also brought us to study the TGF-β pathway in infections with long-lived, essentially tolerogenic, helminth parasites. Finally, within the spectrum of mammalian TGF-β proteins is an exquisitely tightly-regulated gene, anti-Müllerian hormone (AMH), whose role in sex determination underpins the phenotype of freemartin calves that formed the focus of Ray's seminal work on immunological tolerance.

2. The TGF-β superfamily

TGF-β was named for its ability to drive fibroblast proliferation before its broader role in development and immunity had been established; in the meantime related proteins, such as the Bone Morphogenetic Proteins (BMPs) were first characterized in vertebrates before becoming discovered in *Drosophila* flies, *Caenorhabditis elegans* nematodes and other lower animals. Even within the vertebrates, there are more than 30 distinct molecules including three isoforms of TGF-β, Bone Morphogenetic Proteins (BMPs), activins, inhibins, nodal and growth differentiation factors, and anti-Müllerian hormone (AMH) [1–4]. Structurally, these proteins are synthesized as ~400-amino acid inactive pre-proteins, and are cleaved by furin-like proteases to yield an active ~110-amino acid C-terminal domain; the active domain is tightly cross-linked with 3–4 disulfide bonds, and generally forms a homodimer through one additional cysteine residue on each chain.

The ligand family is complemented by a wide-ranging set of receptors, which canonically are heterodimers composed of two serine-threonine receptor kinases, termed Type I and Type II [3]. Depending on the TGF-β family member and expression of appropriate receptors on the surface of cells, these ligands initiate pleiotropic effects in a broad spectrum of biological processes including embryogenesis, immunity, angiogenesis and wound healing [5]. Reflecting this remarkable multiplicity of events dependent on closely related TGF-β proteins, a complex and highly-regulated signaling arrangement exists [6,7].

Regulation of TGF-β signaling takes place in three distinct settings: the extracellular space, the cell membrane and the intracellular region. The full-length TGF-β pro-protein is cleaved to produce not only the *C*-terminal homology domain, but also a functional *N*-terminal 'latency-associated peptide' (LAP) which remains

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0008-8749/

non-covalently attached to the cytokine polypeptide thereby retaining it in inactive form. Prior to secretion from a cell, the LAP-TGF-β complex binds to a further protein, latent TGF-β binding protein (LTBP). Because TGF-β is secreted in this way, as a component of a biologically inactive compound, processes that liberate the active TGF-β molecule can be as important to immunomodulation as its transcription and synthesis [8]. In the extracellular space, the active TGF-β molecule is also prone to sequestration by 'ligand trap' proteins (including LAP), which limit the duration and range of active TGF-β stimulation [9].

In vivo, cell surface receptors including many integrins have the ability to bind LAP-TGF-β and release active TGF-β [10]. This mechanism is of considerable importance for example αv integrin null mice develop similar embryological aberrations to TGF-$\beta^{-/-}$ animals [11]. Furthermore, even if integrin-TGF-β interaction is ablated solely within the dendritic cell compartment, the resultant immunological dysfunction is sufficient to induce spontaneous colitis in normal environmental conditions [12].

Activated TGF-β signals as a homodimer via the union of two type I TGF-β receptors and two type II TGF-β receptors. In humans, 5 variants of the type I receptor and 7 variants of the type II receptor have been identified, in contrast to 29 potential ligands [13]. The affinity of each ligand for individual receptors varies, but most ligands are also able to bind multiple heteromeric combinations of type I and type II receptors, resulting in different downstream effects [13]. Upon binding of the TGF-β ligand, the constitutively active type II receptor is brought into close proximity of the type I receptor, thereby enabling phosphorylation of the TβRI intracellular 'GS' domain and initiating the Smad signaling cascade [14] (Fig. 1).

Smads are intracellular proteins first identified in *C. elegans* worms (as *Sma* genes in small phenotype organisms [15]) and *Drosophilia* flies (as *Mad* genes [16]) and are the key intermediaries in signaling from TGF-β receptors to the nucleus. Hence, not only the ligands but the entire signaling pathway is conserved in the animal kingdom, including parasites such as *Schistosoma mansoni* [17,18].

Eight Smad proteins have been identified in vertebrates and they are sub-categorized according to their function: receptor-activated Smads (R-Smads), common Smads (Co-Smads) and inhibitory Smads (i-Smads) [14]. When TGF-β binds and activates the TGF-β receptor complex, the intracellular GS domain of TβRI phosphorylates Smad2 and Smad3 (R-Smads), which then form a complex with Smad4 (Co-Smad) and enter the nucleus to initiate gene transcription [19]. This process can be regulated intracellularly by Smad7, an inhibitory Smad that can bind TβRI, prevent further signal transduction and then stimulate proteolytic degradation of the receptor [14]. The importance of the i-Smad7 is underlined by observations that it is upregulated in inflammatory bowel diseases, and colitis in mice can be attenuated by antisense Smad7 oligonucleotides [20]. IRF3 is a related transcription factor which can bind and inactivate Smad3; this mechanism may be important in viral infections to suspend TGF-β-mediated immunoregulation until the pathogen is cleared [21].

At the level of the cell membrane, another regulatory mechanism comes into play in the form of 'decoy' receptors such as BAMBI (BMP and activin membrane-bound inhibitor), a transmembrane protein that is structurally very similar to TβRI, but lacks an intracellular GS domain [22]. BAMBI can therefore form a dimer with TβRII and bind TGF-β without initiating any Smad signaling

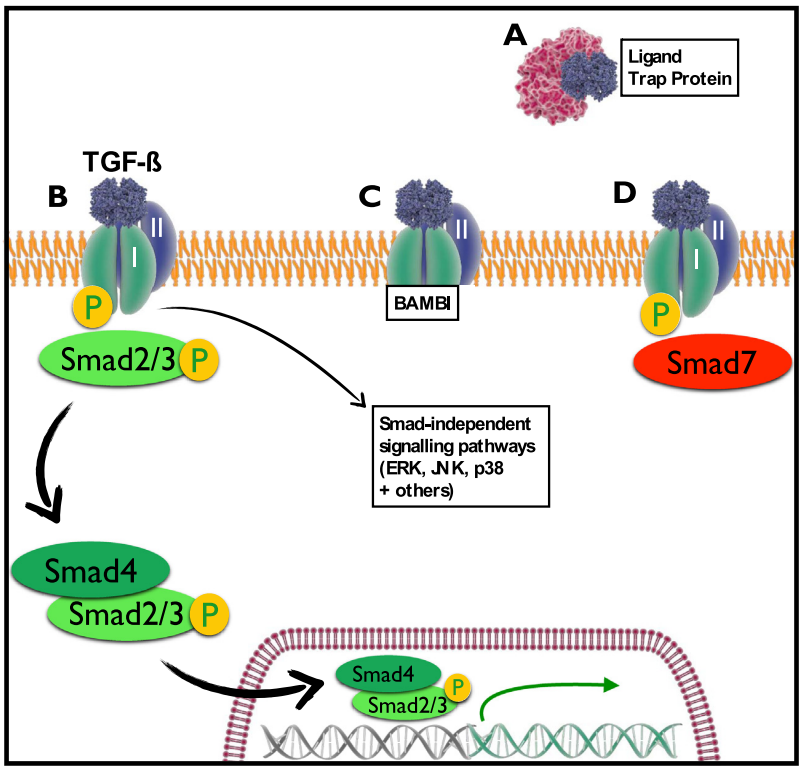

Fig. 1. TGF-β signaling and regulation. (A) Active TGF-β is prevented from binding to receptors due to incorporation with 'ligand trap proteins' such as latency-associated peptide (LAP). (B) Binding of TGF-β to the Type II TGF-β receptor leads to phosphorylation of the Type I TGF-β receptor intracellular domain and activation of the Smad signaling pathway, in addition to Smad-independent signaling including MAPK pathways. (C) Decoy receptors such as BAMBI bind TGF-β but prevent downstream signaling. (D) Smad7, an inhibitory Smad, binds to the phosphorylated Type I TGF-β receptor and prevents downstream signaling.

and thereby reduce the number of TβRIIs available to bind other TGF-β molecules.

The Smad cascade is the 'canonical' signaling pathway for TGF-β and is essential for TGF-β-driven immunoregulation and Treg/Th cellular differentiation [23]. However, TGF-β is also able to activate a number of Smad-independent signaling pathways including mitogen-activated protein kinases (MAPKs). Of these, ERK phosphorylation is an important event in the process of epithelial to mesenchymal transition (EMT), which is necessary in embryological development, but can contribute to pathological fibrosis, one of the major drawbacks in current strategies for therapeutic applications of TGF-β [24].

3. TGF-β in the immune system

TGF-β is a broadly immune suppressive mediator which can, for example, block allergic inflammation in the lung [25] and autoimmune diabetes in the pancreas [26]. Deficiency in either the cytokine or its receptors results in fulminant inflammatory disease that proves lethal in the first weeks of life [27], a phenotype that can be reproduced even if only T cells are unable to respond to TGF-β [28]. The cytokine is instrumental in almost every compartment of the immune system [29], inducing for example B cell class switching to IgA [30,31] and driving myeloid cells into a more tumor-promoting phenotype [32]. But its effects on T cells are perhaps the most prominent, in particular its ability to stimulate naïve $CD4^+$ T cells to differentiate into $Foxp3^+$ Treg that can suppress effector T cell activation and proliferation [33,34], and prolong allograft survival upon adoptive transfer into recipient animals [35]. The level of Foxp3 expression by Treg correlates with functional suppressive capacity [36] and stability of Foxp3 expression is essential for maintenance of a regulatory phenotype [37]. Additionally, TGF-β is capable of promoting a tolerant state through Foxp3-independent mechanisms, such as upregulation of CD73 [38], an ectoenzyme that acts to increase the local extracellular concentration of adenosine.

The combination of TGF-β and certain other cytokines, may induce T cells to differentiate into non-regulatory phenotyopes such as Th17 effectors in the presence of IL-6 [39] and Th9 when IL-4 is present [40]. The action of TGF-β on T cells is antagonized by IFN-γ and IL-4, representing an important pathway by which these cytokines suppress Th17 differentiation [39].

4. TGF-β and regulatory T cells

$CD4^+$ regulatory T cells (Treg), identified by expression of the transcription factor Foxp3, are arguably the single most important cell type in mediating peripheral tolerance [41,42]. Regulatory T cells are subdivided into two types [43]. Thymic Treg (tTreg) constitutively express Foxp3 before leaving the thymus, independently of TGF-β, and play the predominant role of maintaining tolerance to self antigens. In contrast, peripherally-induced Treg (pTreg) develop from naïve, mature $CD4^+$ cells exposed to antigens under tolerogenic conditions (for example by immature DCs with low levels of co-stimulation), and with an essential requirement for TGF-β signaling [44].

Tregs are crucial for physiological immune homeostasis and their absence leads to severe autoimmunity, which is universally fatal in 'scurfy' mice that lack Foxp3 expression [45] and manifests as a life-limiting multisystem disorder in humans – the immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) [46,47]. However, Tregs do also have the potential to cause harm by permitting neoplastic cells to evade anti-tumor immunity [48] and preventing immunity in infections [49]; the long term effects of artificially manipulating Treg populations *in vivo* are therefore unknown.

Other subsets of T cells exert regulatory effects while not expressing Foxp3, through the release of other suppressive cytokines, in particular IL-10 and IL-35 from Tr1 [50] and iTr35 [51] cells. While these have potent down-modulatory functions, TGF-β is unique in being central to both the induction and function of Tregs.

5. TGF-β in transplantation

With the potential to synergise with pro-inflammatory cytokines such as IL-6, TGF-β has the potential to play janiform roles in the context of transplantation [52]. In many experimental animal models, TGF-β-induced immunoregulation, and regulatory T cells in particular, clearly promote tolerogenesis and allograft survival. However, detrimental effects can also arise through interstitial fibrosis as a result of increased myofibroblast differentiation; in addition, the ability of TGF-β to promote Th17 differentiation in the presence of pro-inflammatory cytokines (IL-1β, IL-6) and TLR ligands poses a major threat to transplant acceptance [53]. This clearly cautions against the use of TGF-β activity as a strategy to improve graft survival. However, in the absence of TGF-β, Th17 cells can still develop and indeed are more pathogenic due to enhanced IL-23 receptor expression [54]. Furthermore, higher concentrations of TGF-β are able to overcome the Th17 pathway and restore Treg differentiation [55], arguing that, depending on dose and context, TGF-β may still provide a therapeutic option.

6. TGF-β in infectious diseases

The central immunosuppressive role of TGF-β is also reflected in many infectious diseases, particularly in chronic infections. Helminth parasites, which typically establish themselves as long-term residents in the mammalian host are often associated with both generalized immunosuppression and elevated TGF-β expression [56,57]. Moreover, patients with onchocerciasis [58] and lymphatic filariasis [59] show *in vitro* parasite antigen-specific T cell hyporesponsiveness which can be reversed with anti-TGF-β antibodies. In human helminth infections, IL-10 is also a very prominent immunosuppressive factor [60]; whether this is driven by high levels of TGF-β remains to be ascertained.

Alongside the elevation of TGF-β cytokine, many infectious disease settings are accompanied by expansion of $Foxp3^+$ Tregs [49]. Specifically in the context of helminth parasitism, in murine infections with *Brugia malayi* [61], *Heligmosomoides polygyrus* [62–64] and *Strongyloides ratti* [65] all drive marked increases in Tregs, which in the latter two models can be shown to functionally inhibit the host Th2 protective response and promote chronic infection; moreover, blocking TGF-β signaling in *H. polygyrus* infection results in greater worm expulsion [66], establishing a mechanistic link to this key cytokine. The activity of Tregs is also enhanced in many human helminth infections and, together with other regulatory pathways, may establish a form of immunological tolerance to parasites allowing them to remain in the body for many months or years [67,68].

Several mechanisms may operate to raise TGF-β levels in parasite infection, such as (i) host homeostasis to minimize immunopathology in chronic infection; (ii) pathogen triggering of TGF-β production or activation; or (iii) parasite mimicry of the host cytokine to drive the same pathway as host TGF-β. In fact, examples of all 3 can now be found (Fig. 2).

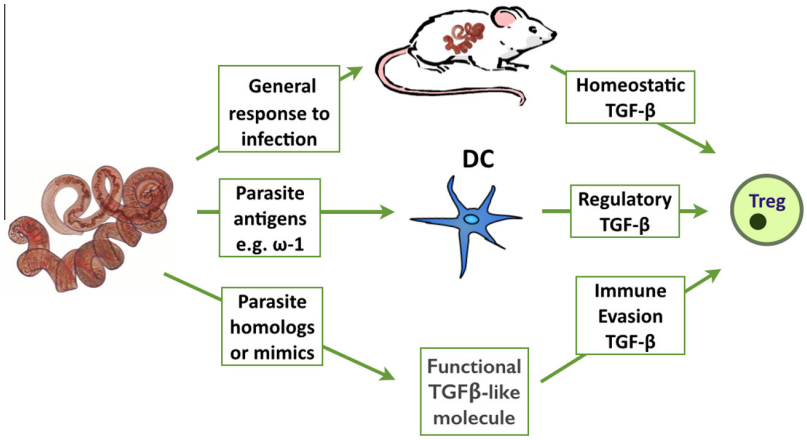

Fig. 2. Helminths and the TGF-β pathway. Several mechanisms may operate to raise TGF-β levels in parasite infection, such as (i) host homeostasis to minimize immunopathology in chronic infection; (ii) pathogen triggering of TGF-β production or activation by host cells such as DCs; or (iii) parasite expression of homologues or mimics of the host cytokine to drive the same pathway as host TGF-β.

7. Host homeostasis or pathogen ploy?

Every immune response must engender a regulatory component that will eventually dampen and terminate that response [69]; hence an increase in TGF-β and Tregs during an infectious episode may reflect host homeostatic mechanisms rather than a strategy evolved by pathogens to suppress immunity.

In a number of parasite models, however, interfering with the TGF-β pathway results in greater resistance to infection; although inferential, these findings are often taken to argue that pathogens benefit from (and may therefore have evolved to favor) TGF-β signaling. These include *in vivo* studies with SB431542, an inhibitor of the ALK5 receptor kinase, [66], and *in vivo* antibody neutralization of host TGF-β [70], each of which lead to greater expulsion of the chronic gastrointestinal parasite *H. polygyrus*.

Interestingly, mice in which T cells express a dominant negative TGF-βRII are not more resistant to helminth infection, but show marked overexpression of inflammatory cytokines such as IFN-γ, blocking an effective type 2 anti-parasite response [71,72]; in parallel, induction of IL-10 is abrogated in infected mice lacking T cell TGF-β signaling, emphasizing the importance of TGF-β in driving other pathways of immunosuppression [71]. Deletion of the TGF-βRII only in myeloid cells, however, results in enhanced immunity to intestinal helminths, indicating that the cytokine may normally inhibit a protective effector myeloid phenotype *in vivo* [73].

Furthermore, while TGF-β levels are elevated in many helminth infections, clearance of parasites through anthelmintic chemotherapy reduces cytokine levels, indicating that the helminths may be actively inducing high expression [74]. Conversely, in the *B. malayi* model system, Foxp3$^+$ Treg expansion required live parasite infection [61]; the inability of dead parasites to induce a regulatory response indicated that Treg expansion is driven by the activity and/or products of live helminths. In some cases, this process may be indirect, as for example in the case of ES of *Trichinella spiralis*, which stimulates host DCs to induce Tregs [75].

8. Parasite induction or activation of host TGF-β

TGF-β levels increase in many different helminth infections, although only in some instances has a causal mechanism been established by which parasites initiate a pathway to ensure cytokine production. For example, in infection with the helminth *S. mansoni*, a regulatory cascade is generated through soluble egg antigens (SEA) that induce T cell differentiation into Foxp3$^+$ Tregs [76]; the effect is sufficient to protect diabetes prone NOD mice from developing autoimmunity. SEA is also capable of suppressing CD4$^+$ T cell proliferation and inducing Foxp3 expression indirectly. Whist SEA-driven Foxp3 induction has only been demonstrated in the presence of DCs (and not culture with CD4$^+$ T cells alone), an increase in the expression of TGF-β latency associated peptide on the surface of CD4$^+$ cells suggests that SEA also induces enhanced secretion of TGF-β by activated T cells, further facilitating Foxp3$^+$ Treg differentiation [77]. In humans, although the mechanism has yet to be defined, helminth infections such as *Onchocerca volvulus* elicit extremely high local levels of TGF-β from many cell types around the nodules in which adult parasite establish themselves [78].

Pathogens may also directly promote activation of TGF-β, as was found in the case of influenza A neuraminidase activating the latent TGF-β complex in epithelial cells and tissues [79]; furthermore, the consequent upregulation of extracellular matrix proteins facilitated the adhesion and infection with streptococcal bacteria, thereby promoting secondary infection of the host.

9. Parasite-derived ligands – homology or mimicry?

We postulated that helminths may express TGF-β homologues that could interfere with host immunity, and characterized several members of the gene superfamily listed in Table 1 along with those identified by other laboratories. In particular, we characterized two ligands in *Brugia* [80,81] and four in *H. polygyrus* [82]. One, *Bm*-TGH-2 from *B. malayi*, was found to activate the MFB-F11 reporter cell line [81], but in unpublished work we found the closest homologue from *H. polygyrus* did not do so. In *Onchocerca*, antibody to mammalian LAP revealed widespread staining of somatic tissues, indicating a parasite-encoded protein sufficiently similar to LAP/TGF-β to be recognized by antibodies [83]. In platyhelminths (flatworms) activin-like homologues have been identified in both *S. mansoni* [84,85] and *Echinococcus multilocularis* [86]; since mammalian Activin A can also stimulate Foxp3 induction and Treg development [87,88], it will be interesting to learn if these parasite ligands can act likewise.

Despite the inability of *H. polygyrus* TGF-β homologues to drive signaling, the proteins secreted by this parasite (termed HES) did directly induce Foxp3$^+$ Treg differentiation in isolated CD4$^+$Foxp3 (GFP)$^-$ T cells *in vitro* with stimulation from Concanavalin A or plate-bound CD3/CD28 [66]. HES also suppressed proliferation of CD4$^+$Foxp3(GFP)$^-$ T cells and promoted IL-17 expression from naïve CD4$^+$ cells when co-cultured with IL-6. This led to further investigation

Table 1
TGF-β ligands in helminth parasites.

Species	Ligands	Properties	References
Ancylostoma caninum	Dbl-1 like, Daf-7 like (TGH-2)	Upregulated in arrested L3 larvae	[93,95]
Brugia malayi	Bm-TGH-1, TGH-2	TGH-2 ligated TGF-β reporter cell line	[80,81]
Echinococcus multilocularis	Activin homolog	Induces Foxp3	[86]
Fasciola hepatica	Fh-TLM and 2 other homologs	Fh-TLM promotes development	[99]
Haemonchus contortus	Hc-TGH-2	Upregulated in L3 larvae	[82]
Heligmosomoides polygyrus	Hp-TGH-2	Upregulated in adult worms and eggs	[82]
Nippostrongylus brasiliensis	Nb-TGH-2	Upregulated in L3 larvae	[82]
Parastrongyloides trichosuri	Daf-7 like	Upregulated in L3 larvae	[94]
Schistosoma japonicum	SjBMP	Ovarian and tegumental expression	[135]
Schistosoma mansoni	SmInAct	Functions in embryogenesis	[84,85]
Strongyloides ratti	Daf-7 like	Upregulated in L3 larvae	[94]
Strongyloides stercoralis	Sst-TGH-1		[96]
Teladorsagia circumcincta	Tci-TGH-1; TGH-2		[96,82]

Table 2
TGF-β family receptors and Smad signaling proteins in helminths.

Species	Receptors	Properties	References
Brugia malayi and Brugia pahangi	Bm-TGR-1, -2		[136]
Echinococcus multilocularis	EnTR1	Interacts with host BMP2	[137]
Schistosoma mansoni	RI + RII	Interact with host TGFβ	[138–140]
Species	**Smad signaling proteins**	**Properties**	**References**
Echinococcus multilocularis	EmSmadA-D	A and C lack MH1 domain	[141,142]
Echinococcus multilocularis	EmSmadE	Phosphorylated by human BMP and TGFβRI	[143]
Schistosoma mansoni	Smad proteins		[144,145]

with a TGF-β reporter cell line (TGF-$\beta^{-/-}$ fibroblasts transfected with a TGF-β-responsive alkaline phosphatase reporter), which confirmed TGF-β activity within HES that could be completely ablated with a type I TGF-β receptor kinase inhibitor, but was unaffected by a pan-vertebrate anti-TGF-β blocking antibody. Thus, HES contains a TGF-β mimic that induces Foxp3 through ligation of the TGF-β receptor complex, but is sufficiently structurally dissimilar to mammalian TGF-β as to be unaffected by a neutralizing antibody. Recently, in work to be published elsewhere, we have isolated the gene encoding a novel protein with no sequence similarity to the TGF-β family, which is able to ligate the mammalian receptor.

Similar expression of Foxp3 in T cells has been reported to be induced by secreted proteins from some other helminths, such as the fox tapeworm *E. multilocularis* [89], and the ruminant nematode *Teladorsagia circumcincta* [66]. Most recently, products from the *Litomosoides sigmodontis* filarial parasite have been shown to ligate the host TGF-β receptor [90]. In each case, the active principles have yet to be determined.

10. TGF-β homologues in helminth arrested development

Originally the TGF-β superfamily gene *daf-7* was found to be a key player in controlling entry into the arrested larval stage of *C. elegans*, the Dauer larvae, which follows the loss of *daf-7* expression [91]; in this model, *daf-7* null mutants constitutively entered Dauer arrest. Parasitic helminths enter crucial and often long-lasting developmental arrest, for example as infective larvae awaiting the opportunity to enter a new host, it was plausible that TGF-β family members might similarly regulate arrest in parasites [80,92]. However, contrary to this prediction, in a number of parasitic species in which TGF-β superfamily homologues were discovered (Table 1), expression was found to be maximal in the arrested third larval instar (L3) stage that is most closely analagous to the *C. elegans* Dauer larva [93–97]. This suggests that either that there has been a functional reversal in the TGF-β signaling pathway between free-living and parasitic nematodes, or that this cascade is not critical to the developmental program of the parasites examined. In a further departure from expectation, the closest *daf-7* homologue in *B. malayi*, TGH-2, is highly expressed in the newborn L1 stage, the microfilaria, which enters arrest in the bloodstream of the host until uptake by hematophagous mosquitos [81]. Whether this reflects an unusual plasticity in the role of TGF-β ligands in nematode development has yet to be investigated.

In platyhelminths, the developmental role of superfamily members is also being analyzed [18,84,98]. However, recent work has screened genomic DNA sequences in the liver fluke *Fasciola hepatica*, identifying 3 homologues, one of which (FhTLM) is able to enhance egg embryogenesis and motility of juvenile parasites when administered as a recombinant protein [99]. Further homologs of the TGF-β receptor superfamily and Smad signaling proteins have also been characterized from several major parasitic helminth species (Table 2).

11. TGF-β homologue in embryonic sexual differentiation

During early development anti-Müllerian hormone (AMH) ablates the Mullerian duct (the precursor of the oviduct) in the male mammal; the only cells expressing AMH are the Sertoli cells which generate high levels of secreted hormone in the developing organism – at 11.5 to 12.5 days post-conception in the embryonic mouse. Amh is secreted at lower levels in the male until puberty and in the female by granulosa cells after puberty. Regulation of AMH is one of the most rigorous examples of control in the genome, and is initiated following expression of the Sry sex determining region on the Y chromosome [100]. The *amh* locus is highly conserved on human chromosome 19 and mouse chromosome 10, adjacent to widely expressed housekeeping genes; hence the *amh* promoter appears to be tightly constrained within a few hundred nucleotides of the start site [101].

Working with the murine Sertoli cell line SMAT-1, expression of AMH was also found to require an enhancer immediately downstream of the 3′ polyadenylation site. At the promoter level it has been found that expression is extremely finely regulated by enhancing (eg GATA1) and inhibitory (eg GATA4) transcription

factors binding to noncoding regions (elements) of the gene which are highly conserved between mammalian species [102–104]. In particular, a high level of expression requires the presence of an enhancer motif immediately downstream of the 3′ polyadenylation site [105]. Mutation of an element within this motif, or of the Wilms tumor element which lies upstream of the gene, ablate high level expression of *amh*; these sites can be considered as anchor points for a specific bridging factor. Remarkably, mutation of a site lying a few nucleotides upstream of the enhancer anchor point leads to an accentuated *amh* expression. Looping between motifs on either side of the coding sequence is necessary for strong activation of the gene.

Interestingly, if cattle conceive a heterosexual pair of twins, placental anastomoses expose the female fetus to the inhibitory effects of AMH in utero, resulting in a masculinized infertile individual known as a freemartin [106]. The other consequence of the anastomosis is hematological chimerism, resulting in establishment of immunological tolerance between the twins, as reported in Ray Owen's landmark study in 1945 [107].

12. New therapeutic strategies?

The potency and breadth of effect of TGF-β ligands suggest many therapeutic scenarios to treat inflammatory diseases and facilitate transplantation. While the non-linear signaling and pleiotropic activities of TGF-β present significant therapeutic challenges, a considerable unmet clinical need currently exists across many severe conditions, and recent advances in understanding have brought the goal of driving immunological tolerance several steps closer. The approaches currently being examined include direct application to dampen inflammation, administration *in vivo* to induce tolerance, and use *ex vivo* to condition patients' T cells into the regulatory phenotype.

Direct administration is currently the least favored strategy, in part because of the pro-fibrotic role of TGF-β which efforts to date have not well dissociated from its immunosuppressive role. There are also concerns that generalized immune suppression resulting from administration of TGF-β might present risks of infection or neoplasia comparable to those of current non-specific immunosuppression regimens.

In recent years, attention has been drawn to the possibility of administering live helminth infections to attenuate or pre-empt inflammatory disorders [108]. From a safety perspective, treatment with low doses of helminth infection may not be hazardous, judging by the millions of people chronically infected with helminths worldwide, of whom very few experience immunological sequelae that approach those of current routine immunosuppression therapy [109,110]. However, the balance between therapeutic efficacy and parasite pathogenicity is not well understood, and is likely to depend not only upon the parasite species in question, but also the genetic predisposition of the host [111], so that adverse effects in a minority of recipients cannot be excluded. Nevertheless, a total of 28 clinical trials of therapeutic helminth infection are now underway or have been completed [112]. While adverse effects do appear to be reassuringly limited, the proposal of experimentally infecting patients with live helminths still engenders a wide range of regulatory, logistical and scientific challenges, such that its unequivocal validation as a beneficial and viable therapy remains elusive [113].

Identification and reproduction of individual helminth-secreted immunomodulatory molecules as potential novel therapeutic agents presents several advantages over live larval therapy [114]. These include consistent pharmacokinetics, scope for pharmacological modification and optimization (reducing immunogenicity of large molecules, for example), improved public acceptability and a lower cost barrier to large-scale production as a routine clinical therapy. Compared with recombinant human TGF-β, it is also likely that helminth-derived homologues have evolved to evade some mechanisms of endogenous TGF-β regulation and may therefore provide the opportunity for greater precision in dosing and specificity of action.

Combination therapies are another approach in development, whereby TGF-β is administered with synergistic 'Treg permissive' agents which may allow some control over downstream cellular differentiation. Of these, rapamycin (sirolimus) can act synergistically with TGF-β to favor Foxp3 expression and Treg differentiation over Th17 effector cells [115], while retinoic acid (RA) is thought to minimize the impact of inflammatory cytokines and co-stimulation on impairing TGF-β-induced Foxp3 expression [116,117].

TGF-β-dependent induction of Treg also occurs *in vivo* and techniques that exploit this mechanism to induce tolerance (such as low dose antigen therapy [118]) appear to generate Treg with more stable expression of Foxp3 than those generated *in vitro* [119]. This suggests that additional stabilizing factors or conditions are present in the *in vivo* setting and might provide encouragement for *in vivo* Treg induction techniques over *ex vivo* expansion and reinfusion of isogeneic cells.

Nevertheless, prevailing concerns about *in vivo* administration of TGF-β are encouraging emphasis on *in vitro* use to generate Tregs which are then administered to patients. In mouse models, adoptively transferred Treg mediate indefinite tolerance of murine allografts including pancreatic islet [120], skin [121] and heart [122]. Expectations of successful translation of Treg therapy into the clinical setting have been high and preliminary clinical trials have now been completed in graft-versus-host disease [123] and hematopoietic stem cell transplantation [124] with modest but encouraging results. To ensure that transfused Tregs are specific for the pathogenic epitope (such as an auto- or allo-antigen), it is also possible to transduce patient T cells *in vitro* with an engineered T cell receptor, creating a highly-targeted and effective regulatory T cell population [125].

However, a number of obstacles and concerns persist. First, Good Manufacturing Practice (GMP)-compliant *ex vivo* expansion of Tregs for subsequent reinfusion is a highly specialized process at a cost of approximately $45,000 per patient [126]. Even if this level of funding could be justified, the infrastructure and highly qualified personnel required are likely to limit translation into routine clinical practice.

Secondly, due to the lack of a unique human regulatory T cell surface marker, accurate identification of Treg populations remains imperfect. The optimal approach is with fluorescence-activated cell sorting (FACS), with selection of, for example, $CD4^+CD25^+CD127^{lo}$ cells [124]. However, GMP-compliant FACS isolation of Treg for clinical therapeutic use is available at very few centers throughout the world necessitating the use of magnetic cell sorting techniques for preliminary clinical trials, with Treg populations consequently of a lower purity [127,128]. Additional steps such as $CD8^+$ T cell depletion can limit alloreactive effector T cells to a very small percentage, but it is likely that any remaining are highly activated, and the long-term impact of their infusion into a transplant recipient is unpredictable [128].

Thirdly, concern remains over the question of whether isolated Treg maintain their regulatory phenotype following re-infusion, particularly in the context of an inflammatory environment. Alloantigen-specific pTreg offer the potential advantages of high functional suppressive ability and a specificity of action that might lower the risk of side effects such as early viral reactivation (observed in trial of Treg therapy in hematopoietic stem cell transplantation [129]) and the potential risk of neoplasia with non-specific Treg therapy. A further caution has been the loss of Foxp3 expression (and therefore regulatory phenotype) once induced

Tregs are no longer exposed to TGF-β [130]. This poses a risk of infusing a population of cells that effectively revert to allograft-specific effector T cells, and the ability or otherwise to treat this scenario with conventional immunosuppression is unknown [131]. Therapeutic infusion of tTreg and pTreg comprise two separate arms of the ONE Study that is currently underway (NCT02129881).

Finally, in the long-term it is unknown whether Treg-mediated immunosuppression might present risks of infection or neoplasia comparable to those of current non-specific immunosuppression regimens. To date, four clinical trials of Treg therapy have been published: three investigating prevention or treatment of graft vs host disease (GvHD) [123,132,133] and one for treatment of type I diabetes [134]. Early follow-up has provided some degree of reassurance, with no adverse events reported other than a slightly increased incidence of viral reactivation in the context of GvHD [129]. However the longest follow-up period that has been reported is only 12 months [134] and, particularly regarding a potential long-term risk of malignancy, it may be very difficult to determine a follow-up period wherein this question can be answered definitively. In short, Treg cellular therapy is an attractive potential therapeutic strategy that has advanced rapidly in recent years, but many questions and logistical barriers still exist, such that translation to routine clinical practice is by no means guaranteed.

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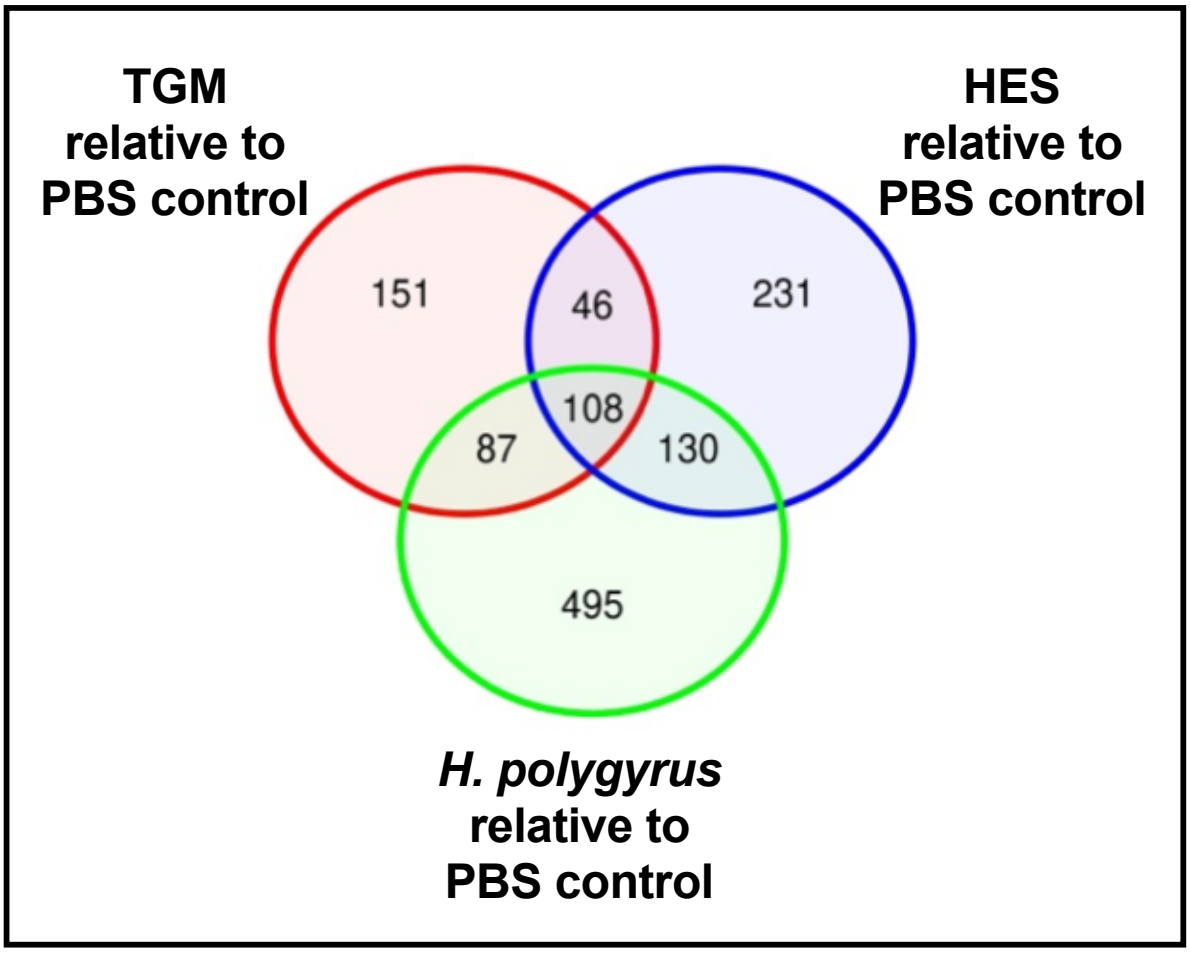
9 Appendix B – Summary of Gene Expression Data from Section 4.2.12 and Figure 4-12



Venn Diagram summarising changes in gene expression of DO11.10 CD4+ T cells following adoptive transfer and immunisation with cognate antigen (pOVA).
Experimental design: figure 4-12; individual gene expression profiles: overleaf.

TGM RELATIVE TO PBS AND HES RELATIVE TO PBS AND *H. POLYGYRUS* RELATIVE TO PBS

Symbol	Description	logFC: TGM relative to PBS	logFC: HES relative to PBS	logFC: HPOLY relative to PBS	Raw p value: TGM v PBS	Raw p value: HES v PBS	Raw p value: *H. polygyrus* v PBS
Mir669b	microRNA 669b	1.903	1.141	1.66	0.0231	0.138	0.0627
Vamp7	vesicle-associated membrane protein 7	1.501	1.024	1.382	0.0819	0.216	0.142
Dcaf13	DDB1 and CUL4 associated factor 13	1.178	1.417	1.252	0.138	0.0813	0.156
Snord69	small nucleolar RNA, C/D box 69	1.147	1.394	2.738	0.369	0.28	0.0729
Mir1967	microRNA 1967	1.088	1.149	1.009	0.287	0.263	0.373
Tfb2m	transcription factor B2, mitochondrial	1.045	1.108	1.249	0.0338	0.0263	0.0253
Mbip	MAP3K12 binding inhibitory protein 1	1.012	1.189	1.459	0.299	0.227	0.188
Kbtbd7	kelch repeat and BTB (POZ) domain containing 7	1.008	1.358	1.245	0.0489	0.0131	0.0328
Mir33	microRNA 33	-1.121	-1.267	-1.533	0.225	0.175	0.145

TGM RELATIVE TO PBS AND HES RELATIVE TO PBS

Symbol	Description	logFC: TGM relative to PBS	logFC: HES relative to PBS	logFC: HPOLY relative to PBS	Raw p value: TGM v PBS	Raw p value: HES v PBS	Raw p value: *H. polygyrus* v PBS
Snord90	small nucleolar RNA, C/D box 90	1.072	3.194	0.334	0.239	0.00407	0.734
Imp4	IMP4, U3 small nucleolar ribonucleoprotein	1.005	1.189	0.979	0.0455	0.0223	0.0743
Imp4	IMP4, U3 small nucleolar ribonucleoprotein	1.005	1.189	0.979	0.0455	0.0223	0.0743
Mir7062	microRNA 7062	-1.007	-1.432	0.502	0.276	0.133	0.618
Scarna9	small Cajal body-specific RNA 9	-1.044	-2.41	-0.384	0.238	0.0163	0.688

TGM RELATIVE TO PBS AND *H. POLYGYRUS* RELATIVE TO PBS

Symbol	Description	logFC: TGM relative to PBS	logFC: HES relative to PBS	logFC: HPOLY relative to PBS	Raw p value: TGM v PBS	Raw p value: HES v PBS	Raw p value: *H. polygyrus* v PBS
Hspa1a	heat shock protein 1A	1.767	0.025	1.784	0.00514	0.961	0.00913
Tmx2	thioredoxin-related transmembrane protein 2	1.368	0.886	1.073	0.0432	0.164	0.135
Mir5104	microRNA 5104	1.232	0.308	1.18	0.159	0.711	0.221
Emc3	ER membrane protein complex subunit 3	1.017	0.962	1.214	0.143	0.164	0.121
Mir15b	microRNA 15b	-1.419	-0.345	-1.375	0.254	0.774	0.319
Mir1b	microRNA 1b	-1.51	-0.708	-1.763	0.027	0.252	0.0223

***H. POLYGYRUS* RELATIVE TO PBS AND HES RELATIVE TO PBS**

Symbol	Description	logFC: TGM relative to PBS	logFC: HES relative to PBS	logFC: HPOLY relative to PBS	Raw p value: TGM v PBS	Raw p value: HES v PBS	Raw p value: *H. polygyrus* v PBS
Mir7-1	microRNA 7-1	0.542	1.063	2.183	0.236	0.0333	0.00114
Gm24148	predicted gene, 24148	0.699	1.525	2.084	0.131	0.00508	0.00143
Snord37	small nucleolar RNA, C/D box 37	0.671	1.028	1.785	0.391	0.2	0.0591
Snora75	small nucleolar RNA, H/ACA box 75	0.412	1.901	1.771	0.68	0.0795	0.135
Klrb1f	killer cell lectin-like receptor subfamily B member 1F	0.777	1.906	1.668	0.241	0.0123	0.0379
Mir7661	microRNA 7661	0.101	1.302	1.503	0.902	0.136	0.125
Isg20l2	interferon stimulated exonuclease gene 20-like 2	0.715	1.069	1.443	0.281	0.12	0.0674
AI464196	expressed sequence AI464196	0.915	1.013	1.313	0.2	0.159	0.109
Gm1818	predicted gene 1818	0.84	1.016	1.177	0.057	0.0265	0.0226
Mir8118	microRNA 8118	0.172	1.504	1.168	0.855	0.132	0.281
Snord16a	small nucleolar RNA, C/D box 16A	0.622	1.059	1.149	0.133	0.0194	0.0223
4631423B10Rik	RIKEN cDNA 4631423B10 gene	0.313	1.014	1.135	0.592	0.104	0.103
A530088E08Rik	RIKEN cDNA A530088E08 gene	0.889	1.166	1.065	0.00366	0.000595	0.00237
Mir6913	microRNA 6913	0.694	1.222	1.03	0.13	0.0159	0.0534
Rnu6	U6 small nuclear RNA	0.807	1.182	1.01	0.36	0.191	0.308

Fads2	fatty acid desaturase 2	0.952	-1.125	1.002	0.195	0.132	0.22
Gm20115	predicted gene, 20115	-0.743	-1.157	-1.023	0.203	0.0606	0.125
Nanp	N-acetylneuraminic acid phosphatase	-0.835	-1.287	-1.034	0.0513	0.00676	0.0342
Olfr530	olfactory receptor 530	-0.672	-1.372	-1.035	0.197	0.0185	0.0866
Snord93	small nucleolar RNA, C/D box 93	-0.209	-1.093	-1.198	0.431	0.00167	0.00191
Mir6915	microRNA 6915	-0.912	-1.205	-1.247	0.0597	0.0187	0.0268
Gstm4	glutathione S-transferase, mu 4	-0.704	-1.083	-1.263	0.164	0.0437	0.0369
Snord98	small nucleolar RNA, C/D box 98	-0.543	-2.18	-3.145	0.7	0.143	0.0677

TGM RELATIVE TO PBS

Symbol	Description	logFC: TGM relative to PBS	logFC: HES relative to PBS	logFC: HPOLY relative to PBS	Raw p value: TGM v PBS	Raw p value: HES v PBS	Raw p value: *H. polygyrus* v PBS
Snord11	small nucleolar RNA, C/D box 11	1.251	0.04	-0.18	0.0458	0.944	0.774
Mir6345	microRNA 6345	1.219	-0.172	0.308	0.12	0.815	0.709
Snord35a	small nucleolar RNA, C/D box 35A	1.17	0.18	0.641	0.159	0.819	0.472
Gm20265	predicted gene, 20265	1.167	0.825	0.868	0.107	0.239	0.265
Snord49b	small nucleolar RNA, C/D box 49B	1.156	0.799	0.9	0.15	0.305	0.302
Cenpn	centromere protein N	1.151	-0.285	-0.013	0.0566	0.604	0.982
Armcx3	armadillo repeat containing, X-linked 3	1.147	0.013	0.867	0.212	0.989	0.388
Nup35	nucleoporin 35	1.142	0.373	0.658	0.0519	0.486	0.281
Il1r2	interleukin 1 receptor, type II	1.085	0.501	0.806	0.327	0.644	0.508
Snora31	small nucleolar RNA, H/ACA box 31	1.073	0.873	0.871	0.0486	0.0976	0.134
Dpagt1	(GlcNAc-1-P transferase)	1.042	0.519	0.915	0.0291	0.233	0.0733
Rab21	RAB21, member RAS oncogene family	1.008	0.932	0.798	0.121	0.148	0.257
Mir5103	microRNA 5103	1.005	0.077	0.027	0.00622	0.797	0.934
Gm10688	predicted gene 10688	-1.014	-0.126	-0.57	0.0464	0.782	0.279
AI606181	expressed sequence AI606181	-1.038	-0.322	0.374	0.0818	0.561	0.546
Trmt11	tRNA methyltransferase 11	-1.069	-0.63	-0.617	0.0114	0.0977	0.141
Mir7651	microRNA 7651	-1.152	-0.983	-0.181	0.106	0.16	0.807
Mir6955	microRNA 6955	-1.337	-0.536	-0.29	0.123	0.515	0.75
Snord82	small nucleolar RNA, C/D box 82	-1.381	-0.15	0.163	0.275	0.903	0.905
Rb1	retinoblastoma 1	-1.456	-0.241	-0.453	0.149	0.801	0.672
Armc8	armadillo repeat containing 8	-1.625	-0.404	-0.253	0.0328	0.551	0.736

HES RELATIVE TO PBS

Symbol	Description	logFC: TGM relative to PBS	logFC: HES relative to PBS	logFC: HPOLY relative to PBS	Raw p value: TGM v PBS	Raw p value: HES v PBS	Raw p value: *H. polygyrus* v PBS
Rnu73b	U73B small nuclear RNA	0.28	2.045	-0.057	0.853	0.196	0.973
Atg14	autophagy related 14	0.301	1.313	0.483	0.563	0.0267	0.412
Prepl	prolyl endopeptidase-like	0.199	1.273	0.291	0.629	0.00988	0.529
Atp11c	ATPase, class VI, type 11C	0.717	1.26	0.957	0.143	0.0193	0.0871
Fas	Fas (TNF receptor superfamily member 6)	0.595	1.252	0.813	0.217	0.0202	0.139
Mir5710	microRNA 5710	0.49	1.233	0.008	0.229	0.00927	0.985
Fam171b	family with sequence similarity 171, member B	0.032	1.197	0.224	0.923	0.004	0.544
Mir3068	microRNA 3068	0.285	1.152	-0.416	0.643	0.0835	0.548
Gm20245	predicted gene, 20245	0.164	1.114	0.863	0.763	0.0613	0.174
Gm6712	predicted gene 6712	0.929	1.11	0.982	0.164	0.103	0.186
Gm19474	predicted gene, 19474	0.219	1.094	0.383	0.839	0.322	0.751
Pop4	processing of precursor 4, ribonuclease P/MRP	0.525	1.06	0.971	0.31	0.0565	0.108

Gm614	predicted gene 614	0.91	1.041	0.973	0.0244	0.0128	0.0298
Mir6898	microRNA 6898	0.039	1.037	0.498	0.94	0.0642	0.392
Gm20285	predicted gene, 20285	0.164	1.03	0.474	0.537	0.00257	0.13
Ift22	intraflagellar transport 22	0.288	1.026	0.009	0.542	0.049	0.986
Snora41	small nucleolar RNA, H/ACA box 41	0.827	1.004	0.941	0.0792	0.0392	0.0747
Rtp4	receptor transporter protein 4	-0.577	-1.092	0.125	0.256	0.0462	0.82
Mir219b	microRNA 219b	-0.908	-1.144	-0.488	0.125	0.0614	0.439
Xlr	X-linked lymphocyte-regulated	-0.712	-1.15	0.626	0.327	0.127	0.436
Ly96	lymphocyte antigen 96	-0.301	-1.155	0.454	0.684	0.14	0.585
Gm19881	predicted gene, 19881	0.146	-1.161	-0.432	0.796	0.0611	0.498
Gm19279	predicted gene, 19279	-0.361	-1.199	-0.161	0.689	0.202	0.872
Kit	kit oncogene	-0.248	-1.214	-0.999	0.702	0.0834	0.187
Tk2	thymidine kinase 2, mitochondrial	-0.855	-1.221	-0.575	0.037	0.00648	0.178
Il17f	interleukin 17F	-0.493	-1.237	-0.711	0.419	0.0613	0.303
Scarna6	small Cajal body-specific RNA 6	-0.714	-1.238	-0.748	0.36	0.128	0.39
Iltifb	IL-10-related T cell-derived inducible factor beta	-0.277	-1.288	-0.179	0.77	0.193	0.866
Actr6	ARP6 actin-related protein 6	-0.613	-1.522	-0.83	0.489	0.105	0.404
Il22	interleukin 22	-0.409	-1.523	-0.054	0.71	0.186	0.965
Snord4a	small nucleolar RNA, C/D box 4A	-0.127	-1.769	0.357	0.898	0.0959	0.747
Mir505	microRNA 505	-0.418	-1.831	0.824	0.766	0.211	0.602

H. POLYGYRUS **RELATIVE TO PBS**

Symbol	**Description**	**logFC: TGM relative to PBS**	**logFC: HES relative to PBS**	**logFC: HPOLY relative to PBS**	**Raw p value: TGM v PBS**	**Raw p value: HES v PBS**	**Raw p value: *H. polygyrus* v PBS**
Mir3066	microRNA 3066	0.112	0.012	2.34	0.853	0.985	0.00532
Mir1929	microRNA 1929	0.566	-0.1	1.936	0.37	0.871	0.0171
Ifi44	interferon-induced protein 44	0.248	0.201	1.697	0.414	0.505	0.000422
Oas2	2'-5' oligoadenylate synthetase 2	0.592	-0.073	1.534	0.187	0.864	0.00839
Ly6c2	lymphocyte antigen 6 complex, locus C2	0.18	0.138	1.524	0.747	0.804	0.0311
Gm4887	predicted gene 4887	0.034	0.45	1.443	0.952	0.435	0.0422
Chil5	chitinase-like 5	0.069	0.341	1.443	0.829	0.301	0.00212
Mir342	microRNA 342	0.552	0.478	1.428	0.522	0.578	0.156
Mir342	microRNA 342	0.552	0.478	1.428	0.522	0.578	0.156
Ly6a	lymphocyte antigen 6 complex, locus A	0.428	0.145	1.427	0.0927	0.542	0.000266
Mir669k	microRNA 669k	-0.013	0.486	1.412	0.977	0.307	0.0194
Parp12	poly (ADP-ribose) polymerase family, member 12	0.121	0.515	1.405	0.761	0.215	0.00923
Purg	purine-rich element binding protein G	0.452	0.487	1.387	0.453	0.419	0.058
Adrb2	adrenergic receptor, beta 2	0.8	-0.048	1.372	0.38	0.957	0.189
Tefm	transcription elongation factor, mitochondrial	0.202	0.07	1.369	0.621	0.863	0.0118
Snord45b	small nucleolar RNA, C/D box 45B	0.368	-0.106	1.359	0.689	0.908	0.203
Snapc1	small nuclear RNA activating complex, polypeptide 1	0.578	0.587	1.344	0.33	0.323	0.0597
Hist1h1c	histone cluster 1, H1c	0.527	0.485	1.329	0.0807	0.104	0.00144
4930421P22Rik	RIKEN cDNA 4930421P22 gene	-0.062	-0.301	1.323	0.724	0.11	4.76E-05
Ifng	interferon gamma	0.909	0.104	1.292	0.0717	0.822	0.0285
Gm9958	predicted gene 9958	0.467	0.541	1.28	0.419	0.352	0.0661
Dnajc19-ps	DnaJ (Hsp40) homolog, subfamily C, member 19	0.708	0.61	1.274	0.145	0.202	0.0293
Bst2	bone marrow stromal cell antigen 2	0.125	0.19	1.258	0.767	0.653	0.021
Tmem203	transmembrane protein 203	0.835	0.823	1.223	0.125	0.13	0.0531
Snora16a	small nucleolar RNA, H/ACA box 16A	0.504	0.992	1.221	0.226	0.0299	0.0194

Gm20305	predicted gene, 20305	0.775	0.286	1.221	0.132	0.558	0.0437
Gnpnat1	glucosamine-phosphate N-acetyltransferase 1	0.942	0.191	1.219	0.0606	0.677	0.0346
Nudt16l1	nudix -type motif 16-like 1	0.579	0.601	1.217	0.298	0.281	0.0663
Usp18	ubiquitin specific peptidase 18	-0.027	0.282	1.205	0.96	0.604	0.0681
Mrps17	mitochondrial ribosomal protein S17	0.439	0.512	1.2	0.275	0.207	0.0183
Brix1	BRX1, biogenesis of ribosomes, homolog	0.733	0.76	1.199	0.179	0.165	0.0606
Rabl3	RAB, member RAS oncogene family-like 3	0.79	-0.16	1.197	0.0695	0.689	0.0205
Gm19601	predicted gene, 19601	0.765	0.305	1.193	0.131	0.526	0.0449
Btg3	B cell translocation gene 3	0.605	0.302	1.187	0.0484	0.287	0.00285
Tmem181a	transmembrane protein 181A	0.807	0.641	1.186	0.422	0.521	0.297
Tmem126a	transmembrane protein 126A	0.605	0.658	1.182	0.18	0.148	0.0307
Gm19486	predicted gene, 19486	0.693	0.372	1.179	0.175	0.451	0.0506
BC094916	cDNA sequence BC094916	0.374	0.23	1.176	0.0876	0.271	0.000365
Gm20053	predicted gene, 20053	0.881	0.748	1.169	0.272	0.347	0.198
Pyhin1	pyrin and HIN domain family, member 1	0.394	0.331	1.165	0.0846	0.139	0.000527
Gm19693	predicted gene, 19693	-0.301	-0.019	1.16	0.518	0.967	0.0443
Nt5dc3	5'-nucleotidase domain containing 3	-0.143	0.594	1.156	0.733	0.177	0.0303
Snord104	small nucleolar RNA, C/D box 104	0.752	0.189	1.148	0.00588	0.398	0.000792
Oas1a	2'-5' oligoadenylate synthetase 1A	0.033	0.094	1.145	0.863	0.631	0.000326
Mir5619	microRNA 5619	0.562	0.108	1.144	0.385	0.865	0.13
Isoc1	isochorismatase domain containing 1	0.281	0.766	1.135	0.672	0.263	0.147
Mir6920	microRNA 6920	-0.688	-0.461	1.131	0.436	0.598	0.261
Ubald2	UBA-like domain containing 2	0.482	0.563	1.123	0.052	0.0276	0.00103
Trav3-3	T cell receptor alpha variable 3-3	0.896	0.614	1.121	0.058	0.173	0.0377
Cycs	cytochrome c, somatic	0.572	0.665	1.115	0.317	0.248	0.0963
Gm10495	predicted gene 10495	0.523	0.159	1.112	0.314	0.754	0.0721
Fam45a	family with sequence similarity 45, member A	0.61	0.89	1.111	0.233	0.0944	0.066
Brcc3	BRCA1/BRCA2-containing complex, subunit 3	0.337	0.591	1.108	0.373	0.134	0.0212
Mir6905	microRNA 6905	-0.012	-0.048	1.099	0.983	0.929	0.0925
Gm19261	predicted gene, 19261	-0.317	-0.241	1.096	0.582	0.675	0.11
Crls1	cardiolipin synthase 1	-0.085	0.031	1.095	0.833	0.94	0.033
Pigf	phosphatidylinositol glycan anchor biosynthesis	0.24	0.276	1.093	0.596	0.544	0.0507
Slamf7	SLAM family member 7	0.804	0.146	1.085	0.0296	0.653	0.0121
Thoc1	THO complex 1	0.465	0.711	1.084	0.115	0.0253	0.00504
Mir6973a	microRNA 6973a	0.794	0.257	1.084	0.0142	0.359	0.00475
Gm4955	predicted gene 4955	0.357	-0.167	1.083	0.264	0.591	0.00949
Snhg5	small nucleolar RNA host gene 5	0.634	0.653	1.079	0.109	0.1	0.0233
Rgs2	regulator of G-protein signaling 2	0.371	0.551	1.068	0.157	0.0469	0.0029
Trav3n-3	T cell receptor alpha variable 3N-3	0.982	0.667	1.067	0.0325	0.122	0.0367
Hprt	hypoxanthine guanine phosphoribosyl transferase	0.51	0.839	1.066	0.44	0.215	0.164
Maea	macrophage erythroblast attacher	0.568	0.518	1.064	0.238	0.279	0.0622
Snora68	small nucleolar RNA, H/ACA box 68	0.505	0.632	1.055	0.155	0.0831	0.0168
Tfb1m	transcription factor B1, mitochondrial	0.794	0.955	1.053	0.191	0.123	0.128
Gm527	predicted gene 527	-0.035	-0.493	1.049	0.924	0.195	0.025
Vbp1	von Hippel-Lindau binding protein 1	0.437	0.759	1.046	0.331	0.106	0.0538
Art2b	ADP-ribosyltransferase 2b	0.211	0.416	1.044	0.73	0.499	0.147
Vkorc1	vitamin K epoxide reductase complex, subunit 1	0.422	0.564	1.04	0.335	0.206	0.0501
Tma16	translation machinery associated 16 homolog	0.677	0.742	1.04	0.144	0.113	0.0544
Amica1	adhesion molecule, interacts with CXADR antigen 1	0.035	-0.052	1.036	0.906	0.858	0.00902

Gm2427	predicted gene 2427	0.468	0.577	1.035	0.259	0.17	0.0397
Uchl5	ubiquitin carboxyl-terminal esterase L5	0.827	0.677	1.031	0.21	0.298	0.166
Ttc32	tetratricopeptide repeat domain 32	0.205	0.098	1.023	0.273	0.59	0.000451
Ighm	immunoglobulin heavy constant mu	0.632	0.097	1.019	0.124	0.803	0.0362
Ciart	circadian associated repressor of transcription	0.282	0.571	1.018	0.179	0.0155	0.000955
Rnu11	U11 small nuclear RNA	0.497	0.384	1.016	0.076	0.157	0.00481
Exosc3	exosome component 3	0.649	0.546	1.016	0.0249	0.0506	0.00425
Ccr2	chemokine (C-C motif) receptor 2	0.859	-0.138	1.015	0.181	0.821	0.16
Rnu3a	U3A small nuclear RNA	0.195	0.508	1.014	0.547	0.136	0.0163
AA414768	expressed sequence AA414768	0.551	0.432	1.013	0.192	0.299	0.0446
Gm4129	predicted gene 4129	-0.165	0.621	1.011	0.557	0.0457	0.00786
Atp6v1b2	ATPase, H+ transporting, lysosomal V1 subunit B2	0.543	0.589	1.009	0.346	0.309	0.132
Ifih1	interferon induced with helicase C domain 1	0.411	0.501	1.005	0.257	0.175	0.0258
Zfp626	zinc finger protein 626	0.485	0.57	1.001	0.321	0.248	0.0835
Snord43	small nucleolar RNA, C/D box 43	-0.04	-0.158	1.001	0.932	0.738	0.0807
Fam26f	family with sequence similarity 26, member F	-0.014	-0.299	1	0.971	0.43	0.0341
Mir1948	microRNA 1948	0	-0.806	-1.063	1	0.2	0.137
Mir687	microRNA 687	-0.17	0.459	-1.092	0.897	0.729	0.466
Mir7212	microRNA 7212	-0.657	-0.26	-1.109	0.277	0.659	0.114
Scarna10	small Cajal body-specific RNA 10	-0.926	-0.714	-1.144	0.347	0.464	0.301
Adam4	a disintegrin and metallopeptidase domain 4	-0.53	-0.9	-1.148	0.298	0.0925	0.06
Mir5123	microRNA 5123	0.326	0.385	-1.211	0.783	0.746	0.37
Gm20274	predicted gene, 20274	-0.063	-0.114	-1.213	0.902	0.824	0.055
Mir7039	microRNA 7039	-0.897	0.21	-1.267	0.3	0.803	0.198
Mir30e	microRNA 30e	-0.896	-0.471	-1.926	0.0975	0.359	0.00574