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Modulation of Dendritic Cells Function by *H. polygyrus* Products

Carried out in Rick Maizels' laboratory

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ABSTRACT

Dendritic cells (DCs) are crucial antigen-presenting cells that can drastically change the development of an immune response by polarising T helper cells toward a Th1 or a Th2 phenotype. *Heligmosomoides polygyrus*, a murine model of human helminth, has been shown to strongly down-regulate its host immune response. At the DC level, its excretory/secretory products (HES) modulate the cytokine response and co-stimulatory marker expression to bacterial stimulation in favour of an anti-inflammatory environment. A regulatory T cell-inducing TGF β -like activity was also discovered in HES.

In this work, we further characterised the immunomodulatory properties of HES and heat-inactivated HES both in vitro, using LPS-pulsed GM-CSF-grown bone marrow-derived DCs, and in in vivo adoptive transfer of HES-, bacterial extract- or co-pulsed DCs. To determine if the TGF β , C-type lectin receptors (CLRs) or toll-like receptors (TLRs) were implicated in this immunomodulation, we treated DCs with blocking antibodies, chemical kinase inhibitors and grew DCs from knockout mice. These studies revealed that HES immunomodulation of DCs was independent of the TGF β receptor, the two CLRs, Dectin-1 and 2, as well as any TLR. Spleen tyrosine kinase (Syk), which is crucial for the signalling of many CLRs, and phosphoinositide 3-kinase seemed not to be needed as well.

In an attempt to reduce the number of potential immunomodulators in HES, HES size fractions were tested for their ability to reduce LPS-induced inflammatory cytokine production of DCs. The activity was limited to few fractions, fraction 14 being the most potent.

Finally, physical interactions between DCs and biotinylated HES were measured by flow cytometry and revealed that CD24, a molecule required for HES interactions with B cells, was implicated, but not crucial. HES binding to CD24^{-/-} DCs was reduced, but the LPS-induced cytokines and co-stimulatory markers levels were still down-regulated upon HES co-treatment.

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1 Introduction

1.1 *Helminth infections*

The word helminth comes from the Greek meaning worms and describes a class of parasitic worms that live inside their host [1]. These parasites can have more or less complex life cycles involving one or two host species. Although, most of the infections by helminths are non lethal, their burden on the populations of developing countries is huge. In order to measure it, the concept of disability-adjusted life years (DALYs) was developed by the World Health Organisation (WHO) in 1990. Each disease was assigned a number of DALYs, which correspond to the sum of the average number of life years infected people will lose due to premature death and the average number of life years infected people will suffer from the disease and therefore lose their productivity. According to the first WHO report on neglected tropical diseases [2] released in 2010, more than one billion human beings are infected by helminths worldwide and these infections are estimated to be responsible for more than 17 million DALYs.

Helminthiases can affect cognitive functions and vision, but also cause deformity and skin diseases. Children are especially affected as they suffer from growth stunting and have difficulties focusing at school. Pregnant women have a higher risk of neo-natal prematurity and pregnancy-associated mortality [1]. All these symptoms are responsible for a high morbidity that prevents poor regions from improvement of their economical situation. Nevertheless, these diseases kill less than the “big three” (AIDS, tuberculosis and malaria) and are confined to the poorest countries in the world as they thrive in low hygiene standards and a tropical climate. This low visibility in developed countries and the lack of interest for the industry to invest in treatments against diseases affecting mostly poor populations resulted in very low funding for research in this topic [2].

A key feature of helminth infection is that, as opposed to bacteria or viruses, worms do not replicate at high rate inside their host. Thus, the intensity of the infection depends on the number of eggs or larvae to which the host is exposed. For the helminths themselves, the critical aspect is their ability to stay alive for a long period inside their host, in order to produce as many eggs as possible. For this purpose, worms must counteract or evade the immune system of their host and some are quite successful indeed. In most cases, immunity to this kind of organism arises only

after several years, showing the incredible ability of helminths to down-regulate their host's immune response.

1.2 Mammalian immune response to helminth infections

Dealing with macro-organism producing eggs has little in common with defence against microorganisms such as bacteria or viruses that replicate at high frequency. Therefore, the mammalian immune system has evolved in order to discriminate different kinds of parasites and set up an appropriate response for each of them. The adaptive T helper (Th) 1-type response is optimized to clear intra-cellular parasite infections. Th1 cells help infected macrophages to kill ingested pathogens and induce the production of immunoglobulin (Ig) G2 by B-lymphocytes. In this setting, cytotoxic CD8⁺ T cells proliferate rapidly to kill infected cells. This type of response is associated with tissue damage and can be more dangerous than helpful if the infection is not quickly contained and cleared. If the pathogen manages to evade the host defences and survives for extended periods, activated immune cells will stay in the infected tissues and the constant exposition to high doses of inflammatory signals will prevent tissue repair and healing. Chronic inflammatory diseases such as autoimmune disease or septic shock, resulting from a cytokine storm, are amongst the adverse effects that can arise from an uncontrolled Th1 response. The Th2-type response is more dedicated to the protection against extra-cellular parasites. In this setting, B cells produce IgE and IgG1, mast cells, basophils and eosinophils are recruited to the site of infection and increased epithelial cell turnover and mucus production are favoured. Thus, The type-2 response is more associated with tissue remodelling and repair [3]. Moreover, smooth muscles are activated and can help to expel intestinal parasites in combination with increased mucus flow [4]. In addition, another type of CD4⁺ T cells recently turned out to be important for mucosal immunity. Th17 have been reported to be involved in the protection against different extra- and intra-cellular bacteria as well as fungal infections [5]. Finally, a negative signal, determined by so-called regulatory T cells (Tregs), is necessary to avoid bursts of inflammatory cytokines, extended tissue destruction and allow the return to a resting state after the pathogen clearance.

Although the Th2 response stays the most efficient way of clearing parasites such as intestinal worms, most helminths require a good balance between Th1, Th2 and the regulatory response in order to be cleared [6]. A strong Th1 response should definitely be avoided, as it implies chronic inflammation, but if the regulatory response dominates, parasites will survive inside the host for an extended period. In

the case of *H. polygyrus* however, the susceptibility of mouse strains seems to be correlated with the ability to quickly mount a strong Th2 response to the worm (unpublished data Filbey et al.).

1.3 The life cycle of *Heligmosomoides polygyrus*

Heligmosomoides polygyrus bakeri is a rodent gut parasite with a fairly straightforward life cycle. The eggs are spread in the environment via the faeces of the host. Thirty-six hours after being laid, eggs hatch, giving way to the first larval stage (L1). Two successive moults, will then give rise to second (L2) and third (L3) larval stages. L3 larvae are infective and can invade the duodenal mucosa if a suitable host ingests them. Two more moults inside the gut mucosa are needed before the parasites can move back into the intestinal lumen as adult worms, about 8 days after infection. Two days later, the first eggs can be detected in the newly infected host's faeces. Altogether, the cycle takes about 13.5 days, but once a rodent is infected, the female worms can survive up to 8 months in the lumen of its duodenum depending on the strain of the host [7] (<http://hpoly.blogspot.com/>). The excretory/secretory products of *H. polygyrus* (HES) are derived from adult worm cultures by filtrating the supernatant in order to collect only the secreted molecules.

1.4 Dendritic Cells and the polarisation of the immune response

Dendritic cells play a central role in the organisation of the immune system, as they provide the crucial link between innate and adaptive immunity. While immature, they sample their environment by taking up antigens through endocytosis. If a danger signal, like pathogen-associated molecular patterns (PAMPs) or molecules secreted during necrosis, for example, is present in the tissue, DCs acquire the ability to migrate to secondary lymphoid organs where they can find and activate CD4⁺ T cells [8]. By doing so, they can polarise naïve CD4⁺ T cells to different types of immune response, including the Th1 response, characterised by interferon (IFN) γ production, the Th2 response, showing a strong interleukin (IL)-4 production, or the Th17 response, defined by IL-17 production. Interestingly, DCs are able to associate one particular response to several different antigens simultaneously, by trafficking them in different cellular compartments [9].

All dendritic cells are derived from bone marrow stem cells, but two main subsets can be distinguished in humans. The CD34⁺ myeloid progenitors give rise to CD14⁻ CD11c⁺ cells, also called "classical" pre-DCs, whereas the CD34⁺ lymphoid progenitors give rise to a CD11c⁻IL3R α ⁺ subpopulation, known as "plasmacytoid"

DCs [10]. While classical pre-DCs are reported to express the Toll-like receptor (TLR) 2 and TLR 4 and produce inflammatory cytokines like tumor necrosis factor (TNF) α , IL-6 and IL-12, plasmacytoid pre-DCs (pDCs) are known to express TLR7 and TLR9 and produce large amount of IFN- α [11].

In the mouse model, similar populations have been reported. Their respective TLR expression remains the same, but classical DCs were described as CD11c^{hi}B220⁻MHCII⁺ and the pDCs as CD11c^{int}B220⁺. In vivo equivalents of these subsets can be obtained by in vitro culture of bone marrow derived DCs (BMDCs), containing either granulocyte macrophage-colony stimulating factor (GM-CSF) which induces TLR4-expressing cDCs [8], or Flt3 ligand (Flt3L) which induces TLR9 expressing pDCs. In different microenvironments, these pDCs are thought to induce Th1-, Th2-type responses, as well as Foxp3⁺ regulatory T cells [10].

In order to activate T cells, DCs must express MHC class II to present antigens to the T cell receptor (TCR), but they must also express co-stimulatory surface molecules, such as CD80, CD86 or CD40 that will provide a co-stimulatory signal to confirm that an immune response must be mounted against the presented antigen [8]. CD40 has been shown necessary to drive Th2 induction in vitro [12] and the expression of these surface molecules can be modified after TLR ligation [13]. Therefore, these molecules are good indicators of the activation state of the dendritic cell and interesting targets for flow cytometry analysis.

In addition, to these two signals, the cytokine cocktail surrounding the T cell can polarise it toward one of the T-helper or toward the regulatory phenotype. Therefore, the cytokines produced by the activated DC, along with those produced by the other innate and adaptive immune cells present in the lymph node at the moment of the antigen presentation, are crucial for the development of an appropriate response (or tolerance) to the presented antigen. It is now known that Th1 response is induced by the release of IL-12 while IL-6 and transforming growth factor (TGF)- β are needed to drive a Th17 response. Unfortunately, no Th2-driving cytokine has been clearly identified until now as IL-4- and IL-10-deficient DCs have still the ability to drive Th2 response [14]. Nevertheless, type-2 immunity is characterized by the release of IL-4, IL-5, IL-9, IL-13 and IL-21. Finally, IL-10 displays strong anti-inflammatory properties [15].

In 2008, Andrew MacDonald and Rick Maizels reviewed three different models to describe the Th1/Th2 polarisation induced by DCs [14]. The first one is the maturation model, in which Th2 response is driven “by default” when DCs are

activated in the absence of a TLR ligand. In the second one, called alternate pathway model, the decision is taken downstream of the pattern recognition receptor (PRR) through a competition between a MyD88-dependent Th1-inducing pathway and a Th2-inducing signalling pathway. Finally, the inhibition model proposes a cross-inhibition between the two signalling cascades. According to this review, the two later models better fit the dynamics of the adaptive immune response and allow its fine tuning. However, experimental evidence is lacking to validate one or more of these models.

Furthermore, the polarisation of the adaptive immune response is not a straightforward process. DCs do not only interact with T cells but probably with a broad range of other immune cells, which could modulate the activation state of DCs throughout the maturation and T cell activation processes. Moreover, the molecular environment of the DCs, which integrate cytokine and chemokine productions from many immune and non-immune cell types, certainly plays a similar role. This is why the authors of this review mention that the Th2-driving signal could possibly come from other cell types.

1.5 Molecular basis for the recognition of foreign molecular patterns

From a molecular point of view, PAMPs can be detected by different pattern recognition receptors (PRRs). These receptors can be cytoplasmic, like nucleotide-binding oligomerization domain-containing protein (NOD)-like receptors, membrane associated, like TLRs and C-type lectin receptors (CLRs), or even secreted. Following PRR activation, the downstream signalling cascade leads to a change in the activation state of the DCs resulting in their migration to the lymph nodes and the involvement of the adaptive immune system. These modifications are relatively easy to detect as they alter cytokine production profile and cell surface marker expression.

Among the pattern recognition receptors, the TLR family is the most studied and best understood. These trans-membrane molecules have the ability to recognise a broad variety of pathogens and pathogenic products. For example, TLR4 can detect lipopolysaccharide (LPS) present on Gram-negative bacteria, whereas TLR9 links unmethylated CpG that are not found on mammalian DNA [16]. TLR signalling activation can also be achieved with small synthetic compounds that mimic PAMPs. Examples are Pam3CSK4 (Pam3), a lipopeptide that binds TLR2 [17], and R848, a known TLR7/8 ligand [18].

Remarkably, members of the TLR family share two main adapter proteins directly downstream of the receptor. These two molecules are called myeloid differentiation primary response gene 88 (MyD88) and Toll/Interleukin 1 receptor (TIR)-domain-containing adapter-inducing interferon- β (TRIF). MyD88 is involved in the signalling of almost all TLRs and most of them are totally dependent on this molecule to induce the activation of the cell [16]. The double-stranded RNA binding TLR3, however, is not dependent on MyD88, but on TRIF only. The TRIF pathway is also an alternative signalling route for TLR4 [19]. The homeostasis of this system is really interesting, as it seems to auto-regulate. On one hand, the recruitment of adaptor molecules such as MyD88 or TRIF results in the activation of the mitogen-activated protein kinase (MAPK) and NF- κ B pathways and therefore in the expression of pro-inflammatory genes. On the other hand, Ojaniemi et al. [20] showed that MyD88 constitutively binds the p85 regulatory subunit of phosphoinositide 3-kinase (PI3K), but that this interaction is further enhanced upon LPS ligation leading to the conversion of phosphatidylinositol (4,5)-bisphosphate (PIP2) into phosphatidylinositol (3,4,5)-trisphosphate (PIP3) by PI3K. Despite the fact that the activation of the PI3K/Akt/mTOR signalling pathway can have both pro- and anti-inflammatory effects, there is growing evidence that, in myeloid DCs, this pathway can prevent an over-activation that could lead to a cytokine storm by down-regulating pro-inflammatory cytokines production and enhancing IL-10 production [21-23]. Moreover, Laird et al. [24] have proposed a complementary negative feedback mechanism according to which the consumption of PIP2 near the activated TLR would reduce the Toll/interleukin-1 receptor (TIR) domain-containing adapter-like (TIRAP) dependent recruitment of MyD88 on TLR4, therefore reducing the activation of the NF- κ B pathway and the inflammatory cytokine production. A simplified schematic of the main molecules involved in the TLR and CLR pathways is available at the end of this document (Appendix Fig. S 9).

A deregulation of this balance in favour of the regulatory pathway would, of course, be very useful for any pathogen. Interestingly, interactions between helminth products and the TLR pathway to diminish inflammation have already been described. Although this paper has been retracted in the meantime, Puneet et al. [25] showed in February 2011 that ES-62, produced by *Acanthocheilonema viteae*, was able to induce the degradation of MyD88 by autophagosomes, therefore limiting inflammatory cytokines production and increasing mice survival in a septic shock model. The PI3K pathway has also been shown to be critical for the down-regulation of LPS-induced IL-12 by another intestinal parasite *Giardia lamblia* [26].

As opposed to TLRs that recognise a broad range of pathogen-associated patterns, classical CLR are specialised in the detection of carbohydrates containing patterns. The binding sites of their carbohydrate recognition domain (CRD) are dependent on Ca^{2+} availability in the extra-cellular environment [27]. Not all CLR act as PRR, but those that do can either induce or inhibit cellular activation. To this end, CLR transmit the extra-cellular signal through their immunoreceptor tyrosine-based inhibitory motif (ITIM) or activation motif (ITAM). Those consensus domains can be found on the cytoplasmic tail of some CLR, like Dectin-1, but other CLR, like Dectin-2 or Mincle, do not possess such a tail. They must, therefore, associate with an adaptor molecule, such as DAP12 or the $\text{FcR}\gamma$ chain that possess immunoreceptor tyrosine-based motives. Upon ligation of the CLR, these motifs get phosphorylated and can then be bound by Src homology 2 (SH2) domain containing enzymes. Most frequently, molecules of the spleen tyrosine kinases (Syk) family bind ITAM domains, leading to the activation of the cell through the NF- κ B pathway. On the other hand, ITIM phosphorylation induces the recruitment of phosphatases that antagonise the activating kinases [28, 29]. However, cases of ITAM mediating inhibition and ITIM mediating activation have also been reported [30].

This ability of CLR to modulate the activation state of the immune cells is of great interest in the search for cellular receptors targeted by *H. polygyrus* in order to manipulate the immune response of its host. The potential role of CLR in this process is supported by the fact that the binding of other helminth products to CLR has already been described. For example, Ritter et al. [31] have shown that schistosomal egg antigens (SEA) can induce IL-1 β through Dectin-2, Syk kinase signalling and Nlrp3 inflammasome activation. Syk is a particularly interesting target as it is widely present in the signalling pathways of the different CLR [32]. However, some CLR, like Dectin-1 can also signal through a Syk independent, Raf-1 dependent pathway [33] (Fig. S 9).

Pathogen associated molecular patterns are not the only type of molecules that can activate the innate immune system. Some non-foreign molecules share this ability. They are called danger-associated molecular patterns (DAMPs) and are up-regulated in case of tissue damage or can be released by activated or necrotic cells. Similarly to PAMPs, DAMPs can bind to TLRs and induce an inflammatory response. However, the cell activation outcome is somehow different in terms of cytokine production and antigen presentation [34]. These differences could be due to alternative co-receptors and accessory molecules in the TLR signalling pathway itself [34], but, in 2009, Chen et al. [35] proposed a new mechanism allowing to

discriminate PAMPs and DAMPs through a specific down-regulation of the response to DAMPs. According to their paper, the ligation of DAMPs, such as high mobility group box 1 (HMGB1) or heat shock proteins (HSPs), by a complex formed by CD24 and a member of the sialic acid-binding immunoglobulin-like lectin (Siglec) family could selectively inhibit the NF- κ B pathway through the interaction of the Src homology region 2 domain-containing phosphatase (SHP) -1 with the ITIM domain of Siglec-G. This model could avoid an inflammation of a sterile wound while allowing a quick inflammation if PAMPs were detected. However, it could also be a good target to hijack for a pathogen trying to keep the immune system in a non-activated state.

1.6 The known immunomodulatory effects of HES

H. polygyrus derived excretory/secretory products are numerous and diverse. A mass spectrometry analysis performed in the lab revealed that no less than 374 different proteins are found in the HES. Nevertheless proteins are not the only components of HES. Carbohydrates, glycans or other small molecules could also be able to modify the immune response to *H. polygyrus*. Moreover, some of these molecules are very abundant, but others can be expressed at low level while remaining biologically active [36]. This makes the molecular analysis of HES a complex task.

Therefore, many research groups have studied the global immunomodulatory effects of HES as a whole. In their paper [37], Segura et al. mention that HES does not induce DC maturation or cytokine production nor modifies antigen uptake and processing. On the contrary, it seems to down-regulate the inflammatory cytokine production of in vitro cultured BMDCs stimulated with different TLR ligands. These results were confirmed in vivo by adoptive transfer of OVA-pulsed BMDC pre-treated with HES. Three weeks after the transfer, the OVA-specific antibody response and the IgE level were significantly reduced in mice that had received HES pre-treated BDMC compared to those that received naïve BDMC. Another remarkable effect described in this paper, is the ability of HES treated BMDCs to induce the generation of IL-10 producing CD4⁺CD25⁺Foxp3⁻ T cells when co-cultured with naïve CD4⁺ T cells. In these cultures, the levels of IL-4 and IFN γ were also strongly diminished. The regulatory function of these CD4⁺ T cells was confirmed by culturing them with effector T cells and the results show that CD4⁺ T cells co-cultured with HES pre-treated DCs were able to reduce the proliferation of effector T cells. Finally, as opposed to other helminth ES products [38], HES was

still able to down-regulate IL-12p70 produced by CpG stimulated BMDCs lacking TLR 2 or TLR 4, arguing against a direct recognition of HES by those TLRs.

In his thesis at Rick Maizels' laboratory, John R. Grainger, confirmed some of Segura's results. He showed in particular that HES pre-treated BMDCs stimulated with LPS in vitro produce less IL-12p70 than naïve BMDCs in a concentration dependent manner. Similarly, HES was able to limit the up-regulation of CD40 and CD86 on the DC surface in this setting. In order to discriminate the effects of heat-labile and heat-stable components of HES, Grainger performed the same experiments with heat-inactivated HES (hiHES). Again, on its own hiHES did not induce any inflammatory cytokines or cell surface markers up-regulation, but the immunomodulatory properties of HES were disrupted by the heat treatment. This result also suggests that there is no endotoxin contamination of the HES as these molecules are heat-stable.

Moreover, referring to Cervi's work [9], Grainger performed an in vivo transfer experiment in which BMDCs were pulsed with HES, hiHES, a bacterial extract from *Propionibacterium acnes* (Pa) or a combination of these products to determine if HES was able to skew the adaptive response to a strong Th1 inducer. Before the transfer, HES but not hiHES, was able to down-regulate the Pa-induced inflammatory cytokine production and cell surface marker up-regulation as previously observed with LPS. Seven days after the transfer, Grainger re-stimulated splenocytes with HES or Pa and observed that HES-pulsed DCs induced a Th2-type response whereas Pa-pulsed DCs induced an IFN- γ /IL-17 rich environment. Heat-inactivated HES showed the same pattern as HES. The co-pulsed groups showed a HES (or hiHES)-specific Th2 and Pa-specific Th1 responses, but no HES-specific Th1 or Pa-specific Th2 responses. Interestingly, the cytokine levels in the co-pulsed groups were much lower than in the groups pulsed with only one antigen with the remarkable exception of HES-specific IL-17 production that reached the same extend as Pa-specific IL-17 in the HES/Pa co-pulsed group.

Grainger also studied the influence of HES on the T cell pool, but his research was focussed on Foxp3⁺ regulatory T cells. Grainger showed that the percentage of Foxp3⁺ T cells was increased in naïve, HES + Concanavalin (Con) A stimulated splenocyte cultures, compared to Con A only treated cultures. Con A is a T cell mitogen needed for Foxp3 induction, as this process requires TCR activation. The suppressive activity of these Foxp3⁺ cells was also assessed and was found to be comparable to natural Tregs.

The main difference with the Segura paper is that Grainger showed that HES, like transforming growth factor (TGF) β , induces Foxp3 without necessity for antigen presenting cells (APCs), by directly culturing Foxp3⁺CD4⁺ T cells in presence of HES. Knowing this, he tried to further characterise this TGF β -like activity. Among other experiments, Grainger used a chemical inhibitor of the activin receptor-like kinase (ALK) 5 (SB431542) since the TGF β signalling cascade depends on the heterodimerisation of TGF β RII with ALK5/TGF β RI. After a pre-treatment with this inhibitor, he added HES or rhTGF β 1 to CD4⁺ T cells and observed no increase in Foxp3 expression in the culture containing the inhibitor. This proved that the Foxp3 inducing activity of the HES is dependent on the TGF β receptor. Although this experiment was performed on T cells, the protocol could easily be adapted to DCs to assess if the TGF β -like activity of HES plays a role in the dendritic cells immunomodulation. Some of Grainger's findings were recently published in the Journal of Experimental Medicine [39].

1.7 Aims of the study

The goal of this project was to characterise the molecular interactions between the excretory/secretory products of *H. polygyrus* and dendritic cells of its murine host. We focussed our research on the TLR4-expressing classical DC subset derived from bone marrow in vitro, as there were several questions to tackle.

- Which components of the HES are implicated in the interactions with the DCs?
- Which molecules on the DC surface are targeted and what role do they play in the immunomodulatory process?
- Which intra-cellular signalling pathway(s) HES is able to hijack in order to modify the gene expression profile of DCs?
- Does the in vitro modulation of the dendritic cell activation profile correlate with an in vivo polarisation of the T helper response upon transfer of these DCs?

On a long-term perspective, the answers to these questions will be a first step toward a better understanding of the global modulation of the mouse immune system by the parasite *H. polygyrus*. This understanding will be an indispensable means to achieve one or both of the two final goals, which are developing vaccines or therapies against human hookworm parasites and discover new

immunomodulatory molecules potentially able to limit autoimmune and allergic diseases.

In our attempt to elucidate these questions, the previously described immunomodulatory properties of HES turned out to be extremely useful. On one hand, HES was modified, specifically depleted for one compound or fractionated and the DC cytokine production was used as a read-out to compare the different versions of the parasite product. On the other hand, the analysis of the HES anti-inflammatory abilities on modified and DCs from knockout mice allowed to research the host side of the immunomodulatory process.

Based on these four questions, this work was split into five parts, the first mainly dealing with the optimisation of the immunomodulation assay used by Segura and Grainger. The three chapters of the second part present our work on three different molecular signalling pathways that we thought could be involved in the regulation of the DC activation state. The third part focusses on our attempt to define molecular targets of interest in the HES, using size fractionation and characterising the effect of calreticulin, a HES component previously described as immunomodulatory [40]. The study of the physical interactions between HES and DCs, carried out with biotinylated HES, is described in the fourth part and, finally, the in vivo transfer experiments are summarized in the last chapter.

2 Materials and Methods

2.1 Animals

C57BL/6, BALB/c and CD24^{-/-} mice characterised by Nielsen et al. [41] were bred and maintained in a specific pathogen-free facility at the University of Edinburgh. Prof. Gordon Brown from the Aberdeen Fungal Group sent us bone marrow from 129SV WT and 129SV Dectin1^{-/-} mice. MyD88^{-/-}, TRIF^{-/-} and MyD88xTRIF^{-/-} on a C57/BL6 background were given by David Gray's laboratory, University of Edinburgh.

2.2 General reagents

2.2.1 Complete RPMI

RPMI 1640 medium, containing HEPES, was supplemented with 2mM L-glutamine, 100U/ml penicillin, 100µg/ml streptomycin and 5% foetal calf serum (FCS). All these compounds were purchased from Gibco.

2.2.2 Bone marrow-derived macrophage culture medium

Dubelcco's Modified Eagle's Medium (DMEM) was supplemented with 20% FCS (Gibco), 20% L929 conditioned medium (produced in the lab), 2mM L-glutamine (Gibco), 100U/ml penicillin (Gibco) and 100µg/ml streptomycin (Gibco).

2.2.3 Hanks' buffer

Hanks' balanced salt solution (HBSS) (Sigma) was supplemented with 2mM L-glutamine (Gibco) and 50mg/ml FCS (Gibco)

2.2.4 *H. polygyrus* culture solution

RPMI 1640 medium, containing HEPES, was supplemented with 2mM L-glutamine, 100U/ml penicillin, 100µg/ml streptomycin, 10mg/ml sterile filtered glucose and 0.1mg/ml Gentamicin. All these compounds were purchased from Gibco.

2.2.5 0.06 M carbonate buffer

45.3ml of Na₂CO₃ 1 M (Sigma) and 18.2ml of Na₂HCO₃ 1 M (Sigma) were added to 936.5ml of distilled water. Then, the solution was adjusted to pH 9.6.

2.2.6 FACS buffer

For flow cytometry, we used phosphate buffer saline (PBS) containing 0.5% bovine serum albumin (BSA) and 0.1% sodium azide.

2.2.7 Ex-vivo media

X-vivo 15 serum free media (BioWhittaker) was supplemented with 2mM L-glutamine, 100U/ml penicillin and 100µg/ml streptomycin.

2.3 Triton X114-mediated LPS depletion

500µl of recombinant *H. polygyrus* calreticulin (HpCRT) were incubated with 1% Triton X114 (Sigma) for 30 minutes at 4°C on a rotating windmill, then 10 minutes at 37°C and centrifuged at 17000g for 15 minutes at room temperature. The aqueous top phase was then carefully harvested and the whole process was repeated two more times. After the last repeat, the aqueous phase was kept at 37°C for 5 minutes and spun a second time at same speed for 15 minutes. No lipid phase was then to see, but, for safety, the bottom 20µl were not harvested. This product was then referred to as LPS-depleted HpCRT.

2.4 *H. polygyrus* life cycle maintenance

Heligmosomoides polygyrus bakeri was maintained in CBAxC57/BL6F1 mice infected with 500 larvae by gavage. After 14 days, the mice were killed and the whole intestines were removed and cut into three sections. The upper section, consisting of the first half of the small intestine, in which worms could be seen, was kept in Petri dishes filled with Hanks' buffer at 37°C whereas the caeca and colons were temporarily kept in empty Petri dishes. Upper sections were opened by cutting the gut lengthways with scissors. Worms were harvested by scraping the inside of the gut wall with a glass microscope slide and the intestinal walls were removed from the Petri dishes. The worm-containing suspension was then incubated in a muslin bag, which allows only motile worms to pass through. To do so, muslin bags were immersed in a Hanks' buffer-filled funnel connected to a plastic tube. This device was kept in a 37°C incubator for about 1 hour until all worms had reached the bottom of the tube.

During this time, *H. polygyrus* eggs-containing faeces were extracted from the colons and caeca. Charcoal was mixed with faeces until reaching a sticky consistency and plated on 5cm diameter dampened filter papers placed in the

centre of Petri dishes. These dishes were then kept in plastic boxes with humid paper to avoid faeces to dry. Larvae could then be collected from these plates each week for about one month by lifting off the filter paper and washing the Petri dish with water. After 3 washes in water larvae were kept at 4°C until infection of mice.

After the hour of incubation, adult worms were collected by disconnecting the plastic tube from the funnel. Worms were washed 6 times in Hanks' buffer and another 6 times under sterile conditions in Hanks' buffer supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (Pen/Strep). Then, the media was complemented with 1mg/ml Gentamicin for 20 minutes before washing the worms another 6 times in Pen/Strep-containing Hanks' buffer. Finally, worms were separated evenly into T25 flasks filled with 15ml *H. polygyrus* culture medium. These flasks were then incubated for 3 weeks at 37°C and the culture media was collected and replaced every 3 to 4 days. Pools of this media were then diafiltrated into PBS over a 3000 MWCO Amicon membrane to derive *H. polygyrus* excretory/secretory products used in the following experiments.

2.5 Cell isolation and culture

2.5.1 Bone marrow-derived DC culture

Femurs and tibias were extracted from mice and kept in ethanol for 3 minutes. Then, bones were washed 5 times in PBS, under sterile conditions. Ends of each bone were cut with a sterile scalpel and bone marrow was flushed out using PBS and a 0.5mm needle. The suspension was then homogenised by repeated passages through a 0.8mm needle. Cells were counted and plated at 2×10^6 cells per bacteriological Petri dish in 10ml complete RPMI supplemented by 20ng/ml GM-CSF (Peprotech) and incubated at 37°C, 5% CO₂ for 10 days. 10ml complete RPMI + 20ng/ml GM-CSF were added to each plate at day 3 of culture. Then, the media was partially replaced at days 6 and 8 by removing 10ml of the culture media from the edge of the plate and adding 10ml fresh RPMI + 20ng/ml GM-CSF. Finally, cells were harvested on day 10 by carefully washing the plates with media.

2.5.2 Bone marrow-derived macrophage (BMDM) culture

To grow macrophages, cells recovered from bone marrow following the protocol described in the previous paragraph were re-suspended at 6×10^5 cells/ml in macrophage-colony stimulating factor (M-CSF)-containing BMDM culture media and 10ml of this suspension were plated per bacteriological Petri dish. The plates were

incubated at 37°C, 5% CO₂ for 7 days and the media was replaced at day 4 by totally removing the exhausted media from the edge of the plate and carefully adding 10ml fresh BMDM culture media. On day 7, the culture media was again completely removed from the edge of the Petri dish. Then, the adherent cell population was carefully washed twice in 2ml warm PBS per plate. Next, macrophages were detached by incubating them for 10 minutes with 2.5ml per plate warm PBS, 3mM EDTA (Gibco), 10mM D-glucose (Sigma). Cells were then harvested with a Pasteur pipette and washed in 40ml warm PBS. Finally, they were re-suspended in DMEM containing 20% FCS, 2mM L-glutamine and Pen/Strep, counted and diluted at 10⁶ cells/ml to be plated at 10⁵ cells per well in the assays.

2.5.3 Adoptive transfer of DCs

Day 10 BMDCs were incubated with 25µg/ml HES, 25µg/ml hiHES or 10µg/ml of heat-inactivated *Propionibacterium acnes* extract (Pa) (gift of Andrew MacDonald laboratory) or a combination of HES and Pa or hiHES and Pa in 6-well plates (2*10⁶ cells/ml in complete RPMI containing 5ng/ml GM-CSF). After 18 hours incubation at 37°C 5% CO₂, cells were harvested and spun down at 400g. Supernatants were frozen for subsequent cytokine analysis, whereas cells were washed twice in PBS to remove the different antigens. Next, cells were re-suspended in PBS at 5*10⁶ cells/ml and then injected into mice intra-peritoneally (i.p.) (100µl cell suspension per mouse). Finally, the remaining cells were stained and analysed for activation markers by flow cytometry.

2.5.4 Draining lymph nodes and splenocytes re-stimulation

Seven days after the DC transfer, spleens and peritoneal draining lymph nodes were mashed through a 70µm nylon filter to create a single cell suspension in ex-vivo media. After 4 minutes in red blood cell (RBC) lysis buffer (Sigma), splenocytes were washed in ex-vivo media, counted and plated in a 96-well plate at 2*10⁶ cells/well in a total volume of 200µl/well ex-vivo media. Lymph node suspensions were directly counted (without RBC lysis) and plated at 5*10⁵ cells/well. Cells were re-stimulated with 2µg/ml anti-CD3 antibody (clone 145-2C11, Biolegend), 10µg/ml HES or 1µg/ml Pa or left in media. After 72 hours incubation, supernatant cytokines levels were measured by ELISA.

2.6 DCs activation assays

In order to assess the immunomodulatory effects of HES, Day 10 BMDCs were counted, diluted at 10^6 cells/ml and plated at 10^5 cells/well in 96-well plates. DCs were stimulated with different TLR ligands, hot alkali-treated zymosan (Invivogen) or the monoclonal anti-CD40 FGK45 antibody produced in the lab by James Hewitson as described previously [42]. LPS (Sigma) was used at a concentration of $1\mu\text{g/ml}$, CpG (ODN 1826, Invivogen) at $10\mu\text{g/ml}$, Pam3CSK4 (Invivogen) at 100ng/ml and R848 (Invivogen) at $1\mu\text{g/ml}$. Depending on the assay, they were simultaneously treated with different concentrations of HES, HES fractions, hiHES or some recombinant proteins. To avoid any time dependent effect, all compounds were plated first and the cells were always added last. HES and hiHES were titrated between $0.05\mu\text{g/ml}$ and $50\mu\text{g/ml}$. Heat-inactivated HES was produced by incubating HES at 95°C for 20 minutes. HES fractions were plated at $10\mu\text{g/ml}$, based on an estimation of their stock concentration and the assumption that the HES proteins were evenly distributed amongst the fractions. Recombinant human TGF β I (rhTGF β) (R&D Biosciences) was used at 0.5, 1 or 2ng/ml and LPS-depleted *H. polygyrus* calreticulin (HpCRT) between 1ng/ml and $10\mu\text{g/ml}$. All these compounds were plated in $50\mu\text{l/well}$ complete RPMI, so that the total volume of each well reached $200\mu\text{l}$.

In the TGF β experiments, few samples of HES were also pre-incubated for 30 minutes with $10\mu\text{g/ml}$ of a pan-vertebrate anti-TGF β antibody (clone 1D11). In addition, for some experiments, BMDCs were treated with monoclonal blocking antibodies for 30 minutes before the activation. Anti-Dectin-1 antibody (clone 218820, R&D) was used at $3\mu\text{g/ml}$, according to the datasheet. Anti-Dectin-2 (clone D2.11E4, AbD Serotec) was used at various concentrations between 2 and $100\mu\text{g/ml}$.

For chemical inhibitions, BMDCs were pre-treated for 30 minutes with less than 1/500 volume/volume DMSO containing or not the inhibitor. ALK5 was blocked with a final concentration of $5\mu\text{M}$ SB431542 (Tocris Bioscience). Spleen tyrosine kinase inhibitors ER 27319 maleate (Tocris Bioscience) and BAY 61-3606 (Santa Cruz Biotechnology) were titrated between 10nM and $10\mu\text{M}$. PI3K was inhibited with 10nM , 100nM or $1\mu\text{M}$ wortmannin (Calbiochem).

Finally, the plates were incubated for 18 hours at 37°C and 5% CO₂, then spun at 400g for 2 minutes. The supernatant was collected for cytokine detection and cells were stained for flow cytometry analysis.

2.7 Detection of cytokines by ELISA

Enzyme-linked immunosorbent assay (ELISA) was used to detect levels of cytokines in the culture supernatants. ELISA plates (Immunoplate MaxiSorp, NUNC) were first coated with capture antibody at concentrations stated in Table 1 in carbonate buffer (50µl per well). The plates were then incubated overnight at 4°C.

The next day, the plates were washed five times with TBST (TBS + 0.05% Tween) and then blocked with 200µl per well TBST + 10% FCS during two hours at 37°C. The plates were then washed another five times with TBST before adding standards and samples (50µl per well). Standards were made up in complete RPMI by serial doubling dilution from the top concentrations indicated in table 1. Again, plates were incubated at 4°C overnight.

On the third day, plates were washed five times in TBST. Then, the biotinylated detection antibody was added at the appropriate concentration (see Table 1) in TBST + 5% FCS (50µl per well) and the plates were incubated for one hour at 37°C. After five additional washes in TBST, Extravidin alkaline phosphatase (AP) (1/10000) or Streptavidin-horseradish peroxidase (Strep-HRP) conjugate (1/1000) (both from Sigma) were added as developing enzymes in TBST + 5% FCS (50µl per well) and plates were incubated for another 45 minutes. Finally, plates were washed five times in TBST and two times in ultra pure water. Extravidin AP was revealed by 50µl/well p-nitrophenyl phosphate (pNPP) substrate whereas 50µl/well 1:1 ABTS reagent (KPL) were used to develop Strep-HRP-coated wells. The plates were then incubated in the dark until colour appeared. Finally, the optical density (OD) of each well was measured at 405nm with a precision microplate reader (Molecular Devices).

	Capture Ab Conc.	Top Standard Conc.	Detection Ab Conc.	Developing Enzyme
IFN-γ	2 μ g/ml	50 ng/ml	1 μ g/ml	Extrav. AP
IL-4	2 μ g/ml	8 ng/ml	1 μ g/ml	Extrav. AP
IL-5	1 μ g/ml	10 ng/ml	1 μ g/ml	Extrav. AP
IL-6	2 μ g/ml	25 ng/ml	0.5 μ g/ml	Extrav. AP
IL-10	4 μ g/ml	10 ng/ml	2 μ g/ml	Extrav. AP
IL-12p40	2 μ g/ml	50 ng/ml	0.5 μ g/ml	Extrav. AP
IL-12p70	4 μ g/ml	50 ng/ml	0.5 μ g/ml	Extrav. AP
IL-13	2 μ g/ml	10 ng/ml	0.5 μ g/ml	Extrav. AP
IL-17	0.5 μ g/ml	20 ng/ml	250 ng/ml	Extrav. AP
TNFα	0.8 μ g/ml	4 ng/ml	150 ng/ml	Strep-HRP
	Capture Clone and Supplier	Recombinant cytokine	Detection Clone and Supplier	
IFN-γ	R46A2, in house	BD	XMG1.2, BD	
IL-4	11B11, in house	BD	BVD6-24G2, BD	
IL-5	TRFK5, BD	BD	TRFK4, BD	
IL-6	MP5-20F3, BD	BD	MP5-32C11, BD	
IL-10	JES5-2A5, BD	BD	SXC-1, BD	
IL-12p40	C15.6, BD	R&D	C17.8, BD	
IL-12p70	9A5, BD	R&D	C17.8, BD	
IL-13	38213, R&D	R&D	Polycl. rabbit IgG, Peprotech	
IL-17	TC11-18H10, BD	R&D	TC11-8H4.1, BD	
TNFα	Polycl. goat IgG, R&D	BD	Polycl. goat IgG, R&D	

Table 1 - Technical specifications of ELISA antibodies

BD stands for BD Pharmingen™, R&D stands for R&D Systems.

2.8 Biotinylated lectins ELISA

To detect glycans in the different HES fractions, ELISA plates (Immunoplate MaxiSorp, NUNC) were first coated with 1 μ g/ml total HES or the estimated equivalent protein amount of each HES fraction in 50 μ l/well carbonate buffer. After an overnight incubation at 4°C, plates were washed 5 times in TBST and blocked for 2 hours at 37°C with 200 μ l/well TBST 0.5% casein (BDH Chemicals). After 5 washes in TBST, the seven biotinylated lectins of the kit I from Vector Labs were diluted at 2 μ g/ml in TBST 5% FCS and 50 μ l/well were plated and incubated for 2 hours at 37°C. Plates were then washed 5 times in TBST and Strep-HRP was added at 1/1000 in 50 μ l/well TBST 5% FCS for a 30 minutes incubation at room

temperature. Finally, plates were washed, developed and read as described in the previous section.

2.9 Flow cytometry

A subset of cells from each culture was kept for flow cytometry analysis. Cells were first centrifuged at 400g for 5 minutes, then re-suspended in FACS buffer, spun again and blocked in 500µl/Eppendorf or 30µl/well in a 96-well plate rat IgG FcR block (1/40) for 5 minutes at 4°C. Then, cells were washed in FACS buffer, before staining them for 20 minutes at 4°C. A maximum of 2×10^6 cells were used for each stain. All fluorophore-attached antibodies (see table 2) were used at 1/200 dilution in 200µl/Eppendorf or 20µl/well staining solution except for anti-Dectin-1 antibody that was used at 1/10 and MHCII-PerCP antibody that was used at 1/400. After the staining step, cells were washed twice in FACS buffer and directly flowed through the FACS analyser or fixed in 2% paraformaldehyde (PFA) for a later use.

Target	Colour	Clone	Company
B220	PerCP	RA3-6B2	Biologend
CD11b	Pacific Blue	M1/70	Biologend
CD11c	APC	N418	Biologend
CD11c	Pacific Blue	N418	Biologend
CD11c	PE	N418	Biologend
CD24	PE-Cy7	M1/69	Biologend
CD4	FITC	GK1.5	Biologend
CD40	FITC	3/23	BD
CD40	PE	3/23	BD
CD8	FITC	53-6.7	Biologend
CD80	PE	16-10A1	BD
CD80	APC	16-10A1	Biologend
CD86	PE	GL-1	BD
CD86	Alexa Fluor® 488	GL-1	Biologend
Dectin-1	APC	2A11	AbD Serotec
F4/80	Alexa Fluor® 488	BM8	Caltag
F4/80	PerCP-Cy5.5	BM8	Biologend
Gr-1	PerCP	RB6-8C5	Biologend
MHC class II	FITC	M5/114.15.2	Biologend
MHC class II	PerCP	M5/114.15.2	Biologend
Streptavidin	APC	Streptavidin	Biologend

Table 2 - Technical specifications of flow cytometry antibodies

For the biotinylated HES binding experiments, the same protocol was used, but 20µl of FACS buffer with or without biotinylated HES at final concentrations ranging from 0.25 to 4 µg/ml were added to the cells during the main staining step. Then, a second staining step of 15 minutes at 4°C followed by two supplementary washes was performed to allow for streptavidin-biotin binding.

Flow cytometry analyses were performed with a FACS Canto or a LSRII cytometer (BD Bioscience) and the FlowJo software (TriStar).

2.10 Two dimensional electrophoresis

In a first step, proteins were separated by charge by isoelectric focusing (IEF), using the Ettan IPGphor II IEF System. To this end, 8µl IPG Buffer pH 3-10 (GE healthcare) and 50mg 1,4-dithioerythriol (DTE) (Fluka) were added to a 1ml aliquot of rehydration buffer (7 M urea (GDH), 2 M Thiourea (GDH), 4% CHAPS (Sigma) and trace of bromophenol blue (Sigma)). 25µl of each sample were then mixed with 100µl of this solution and spread on an IEF strip holder. A 7 cm Immobiline™ DryStrip pH 3-10 (GE healthcare) was then loaded on the strip holder and topped with PlusOne DryStrip cover fluid (Amersham Bioscience). The holders were then placed on the Etan IPGphor II for isoelectric focusing.

On the next day, IEF strips were equilibrated and the size separation was achieved by SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis). Each strip was first incubated on a rocker for 15 minutes with 2ml of strip equilibration solution (1.5 M Tris pH 8.8, 6 M urea, 30% glycerol, 2% SDS, and trace of bromophenol blue) supplemented with 20mg DTE followed by 15 minutes with 2ml strip equilibration solution supplemented with 80mg iodoacetamide (Sigma). Strips were then loaded on NuPAGE 4-12% Bis-Tris ZOOM gel (Invitrogen), topped with a hot 0.5% w/v agarose solution (in MES SDS running buffer (Invitrogen) with trace of bromophenol blue) and ran in MES SDS running buffer at 120V for one hour.

2.11 Silver staining

To reveal the proteins on the 2D-gels, the Amersham Mass Spectrometry safe silver staining method was used. The gels were successively incubated for 30 minutes in fixation buffer (40% ethanol (Fisher Scientific), 10% glacial acetic acid (Fisher Scientific) in double-distilled water) and 30 minutes in sensitising buffer (30% ethanol, 5% w/v sodium thiosulphate (Sigma), 0.8 M sodium acetate (Sigma) in double-distilled water), then, washed three times 5 minutes in distilled water. They

were then stained in a 2.5% w/v silver nitrate (Sigma) solution (in double-distilled water) for 20 minutes in the dark and washed twice for one minute with double-distilled water. Finally, the gels were incubated in developing buffer (200mM sodium carbonate (Sigma), 0.01% formaldehyde (Sigma) in double-distilled water) for about 5 minutes until the apparition of clear dots. At that point, the reaction was stopped with 40mM EDTA-N₂Na₂.2H₂O in double-distilled water.

2.12 Western blot-like detection of glycans

In order to detect glycans on the HES compounds, 2D-gels were transferred onto a nitrocellulose membrane (BioRad) for western blotting. To this end, a classical sandwich configuration was set up in a semi dry transfer apparatus and the device was run for 90 minutes at 35V in NuPAGE transfer buffer (Invitrogen). The nitrocellulose membrane was then blocked in TBST 0.5% BSA for one hour at room temperature on a rocker before an overnight incubation at 4°C with biotinylated concanavalin A diluted at 2µg/ml in TBST 0.5% BSA. On the next day, membranes were washed twice for 10 minutes in TBST and incubated one hour at room temperature with Strep-HRP diluted at 1/1000. After two additional 10 minutes washes in TBST, bound Con A was revealed by covering the membrane with the enzyme substrate (a 1:1 mix of ChemiGlow Luminol/Enhancer Solution and ChemiGlow Stable Peroxide Solution (Cell Biosciences)) for 5 minutes. The gels were then read on a GelDoc device (Alpha Innotech).

2.13 Statistical analysis

Student's T tests were performed with Prism 5.0c (Graphpad Software Inc.) to compare pairs of groups. Two-tailed P values smaller than 0.05 were considered significant.

3 Results

3.1 *In vitro* immunomodulation of BMDCs by *H. polygyrus bakeri* ES

Before starting to ask how *H. polygyrus* is able to modify the behaviour of DCs as reported by Segura [37] and Grainger [39], we needed to choose the best protocol for growing our BMDCs *in vitro* and then make sure that the reported immunomodulation could be achieved. As this project is built on top of John Grainger's work in the Maizels laboratory, it was logical to keep his protocol for growing BMDCs. However, his method takes ten days and, aiming for efficiency, we decided to compare it with a seven days protocol in which media were changed on days 3 and 6 and cells were harvested on day 7. First of all, we compared the outcome of the cell cultures after 7 or 10 days by flow cytometry, looking for their expression of CD11c, MHC class II, and contaminations of T cells (CD4 and CD8), B cells (B220) or granulocytes (Gr-1) which are all cell types that can be derived from bone marrow progenitors. We immediately noticed that the percentage of BMDCs was higher and the Gr-1 expressing population smaller after ten days of culture (Fig. 1). Moreover, a CD4 positive population was still present after 7 days, but had totally disappeared after 10 days of culture. This figure also shows that it is possible to reach about 90% of CD11c positive cells with the ten days protocol as opposed to only 60% after seven days.

Regarding the co-activation markers, we determined that the naïve day 10 BMDCs express high levels of CD80, intermediate levels of CD86, but nearly no CD40 (Fig. 2). All these markers as well as MHC class II were up-regulated upon LPS ligation, but the addition of HES in the culture medium reduced this augmentation in a dose-dependent manner (Fig. 3), thus confirming Grainger's data. During the whole project, the phenotype of BMDCs was checked for every single culture and, if DCs expressed abnormally high levels of CD40 and CD86, the culture was discarded.

In addition, we had to optimise the assay used by Segura and Grainger and try to get the strongest HES immunomodulation, so that potential intermediate effects could be seen more clearly in future experiments. Therefore, we co-treated Day 7 and Day 10 BMDCs with different TLR ligands, including LPS used by Grainger and CpG used by Segura, along with different HES concentrations and looked for the cytokine level modification in the culture supernatant 18 hours later. This timing, also chosen by Grainger, allows the DCs to reach their maximal production of IL-

12p70 and $\text{TNF}\alpha$ [43], two cytokines that could be useful read-outs for our experiments. As expected, IL-12p70 showed the best signal to noise ratio with a very strong suppression induced by co-treatment with more than 5 $\mu\text{g/ml}$ HES (Fig. 4). $\text{TNF}\alpha$ down-regulation was also efficient. IL-6 and IL-12p40 seemed to show a slight down-regulation with high doses of HES whereas IL-10 level stayed quite stable upon HES addition. Concerning TLR ligands, Pam3 did not induce IL-12p70 and was therefore rejected. As CpG and LPS showed similar patterns, we chose to perform our next experiments with LPS for financial reasons, but also because the cytokines levels seemed more consistent amongst the triplicate wells. Finally, following these results, we decided to use HES at a concentration of 10 $\mu\text{g/ml}$ in the next assays to ensure the most efficient immunomodulation, while sparing this precious product.

The comparison of the two culture protocols revealed that the IL-6 and IL-12p70 immunomodulation was conserved, but Day 7 cells did not change their IL-12p40 production in response to HES treatment and IL-10 seemed to be weakly down-regulated by HES in the Day 7 setting. More importantly, LPS and CpG induced around 6 times more IL-12p70 on Day 10 DCs than on Day 7 DCs. This, along with the phenotype differences described earlier, convinced us to retain the Day 10 protocol for the rest of the project.

To complement Grainger's study on heat-inactivated HES, we decided to treat LPS activated BMDCs with a titration of this product. The goal was to confirm that hiHES couldn't down-regulate the inflammatory cytokines production of DCs. Surprisingly, the level of IL-12p70 was in fact lower in the LPS/hiHES co-treated group than in the LPS treated group, but this effect was independent of the hiHES concentration (Fig. 5). This result clearly shows that the immunomodulatory properties of HES are heat-labile, but opens the question of a parasite effect due to the boiling procedure that could for example modify the interactions of some HES components with the plastic walls of the Eppendorf tubes or even induce the release of some molecules of the tube in the solution. To assess the effect of the boiling procedure, we treated LPS pulsed DCs with boiled PBS and noticed the same kind of intermediate cytokines levels (Fig. 6). Thus, even if this result seems to confirm that hiHES by itself does not change the cytokine production, it also indicates that the heating procedure was not optimal. Nevertheless, in regards of all the data collected during the project, boiling HES seems to disrupt dramatically its ability to down-regulate inflammatory cytokines production, suggesting that heat-labile components, like proteins, are crucial in this process.

Another issue was the timing of the addition of HES in the medium relatively to the TLR ligation. In his studies, Grainger treated the DCs with LPS and HES simultaneously, whereas Segura pre-treated the cells with HES for 2 hours before the addition of CpG. As both settings seemed to work, we decided to further investigate the ability of HES to down-regulate the inflammatory cytokine production even after the TLR ligation. Surprisingly, HES turned out to be even more potent than expected, suppressing nearly totally IL-12p70 production and still down-regulating other inflammatory cytokines levels when added to the culture medium up to 8 hours after LPS (Fig. 7). These results also confirmed that a simultaneous treatment with HES and the TLR ligand was perfectly efficient on top of being the simplest way of setting up the assay.

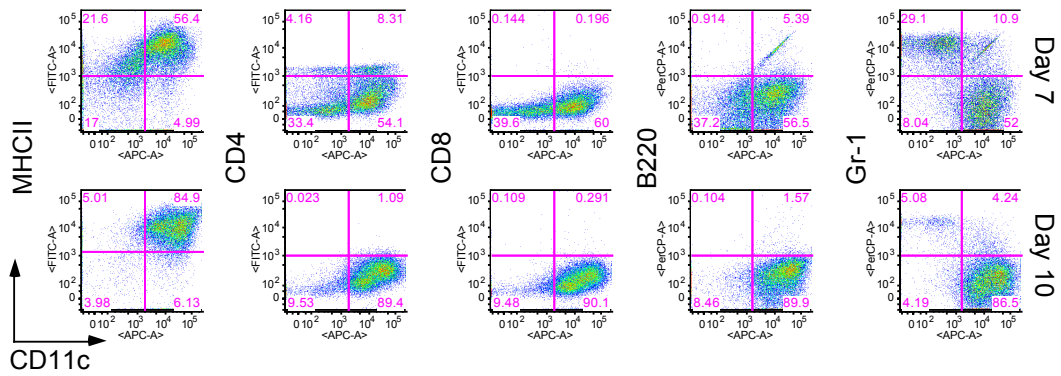


Figure 1 - Comparative flow cytometry analysis of day 7 and day 10 naïve BMDCs.

Naïve BMDCs grown either 7 or 10 days in complete RPMI + 20ng/ml GM-CSF were stained for their surface expression of CD11c, MHC class II, CD4, CD8, B220 and Gr-1. Using the forward and side scatter, dead cells were gated out. Positive gates were chosen in accordance with the single stains and unstained histograms for each fluorophore.

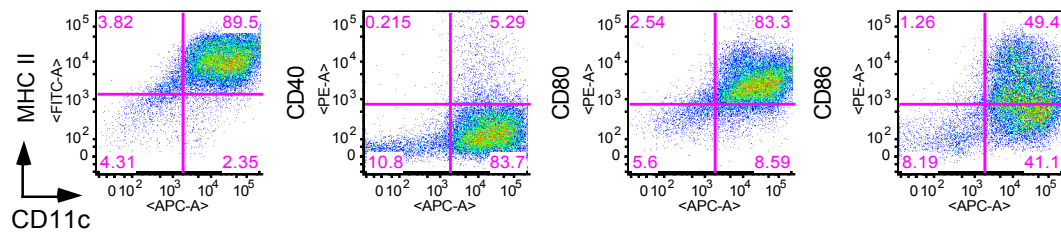


Figure 2 - Example of a flow cytometry analysis of day 10 naïve BMDCs.

As for figure 1, DCs were stained and live cells were gated for their surface expression of CD11c, MHC class II, CD40, CD80 and CD86. Representative plots of the majority of the cultures are displayed.

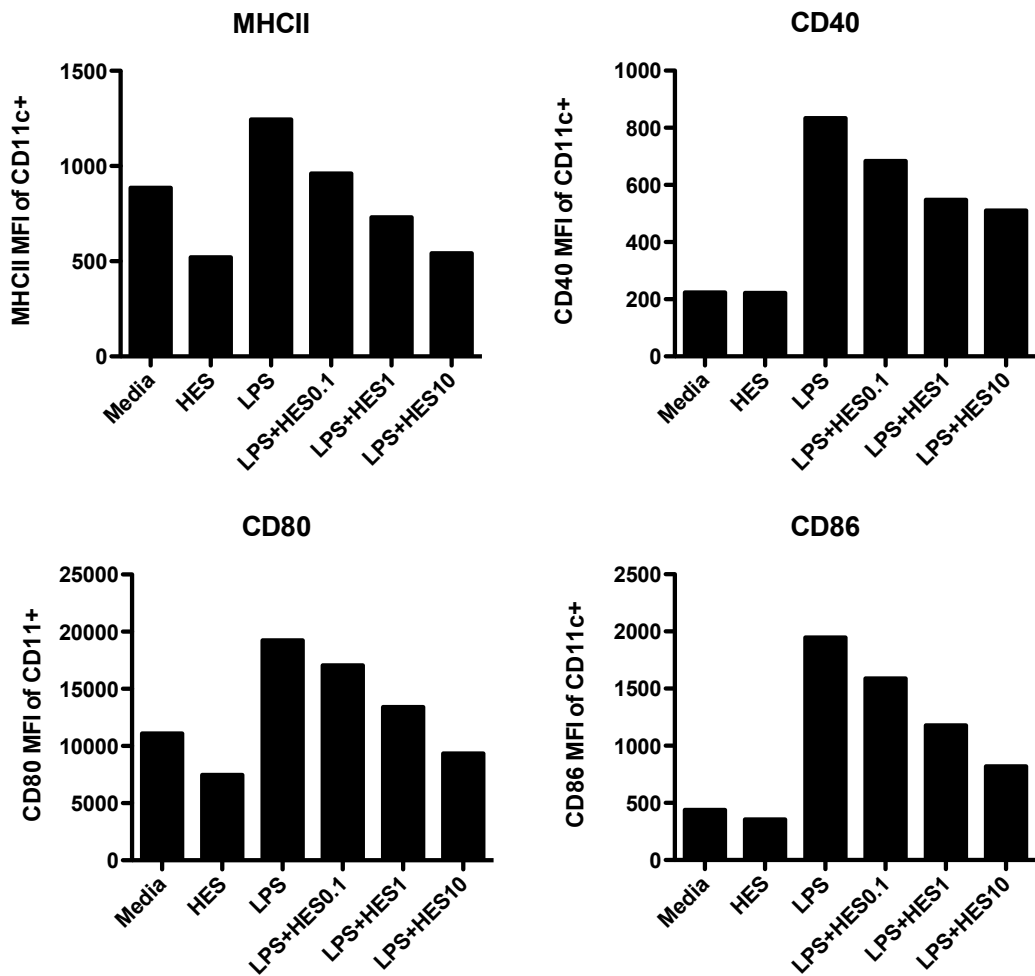


Figure 3 - HES limits the up-regulation of co-stimulatory surface markers in response to TLR4 ligation.

Day 10 BMDCs were either stimulated for 18 hours with 10 μ g/ml HES or 1 μ g/ml LPS with or without HES (0.1 μ g/ml, 1 μ g/ml or 10 μ g/ml) or kept un-stimulated during that time. DCs were then stained for CD11c, MHC class II, CD40, CD80 and CD86 and gated for live and CD11c positive cells. The geometric mean of the fluorescence intensity was then calculated for each marker. Representative plots of several experiments are displayed. In this experiment, single wells were analysed and no statistics are therefore available.

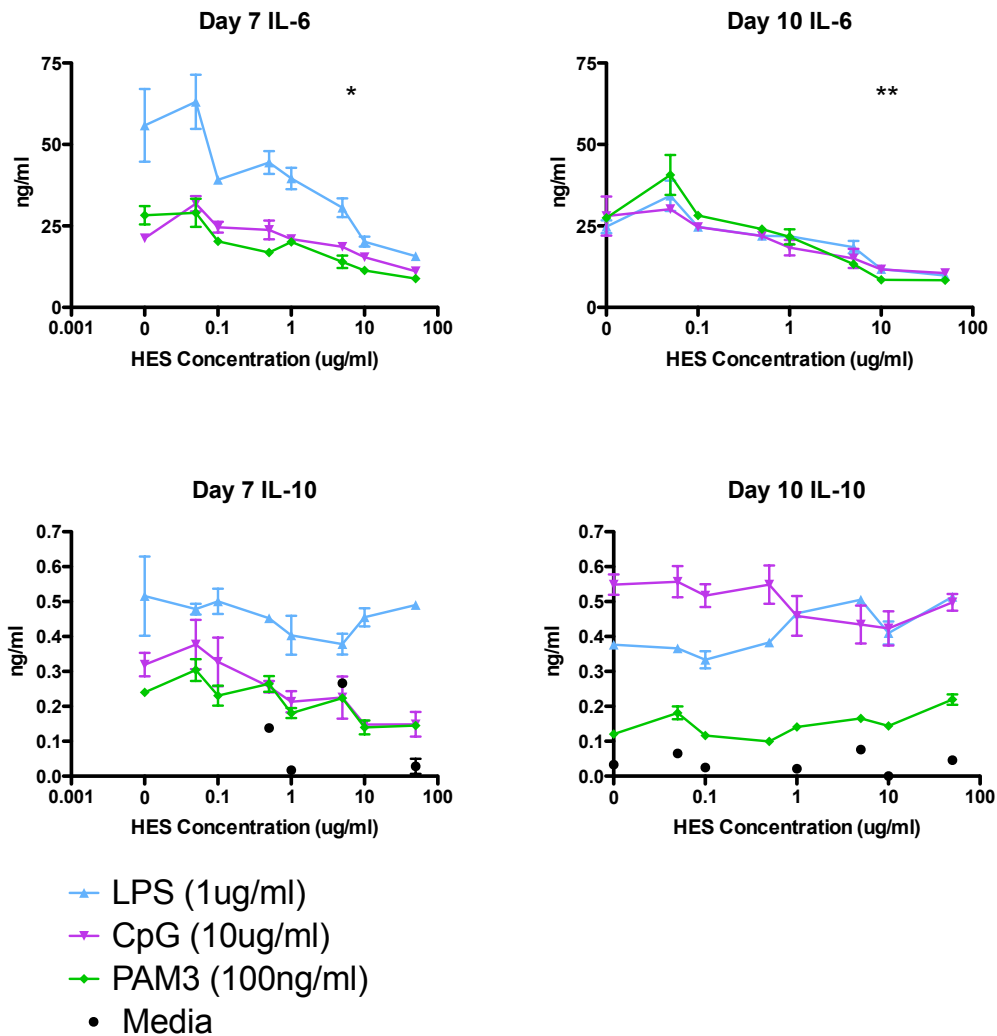


Figure 4 Part 1 - HES down-regulates IL-6, IL-12p40, IL-12p70 and TNF α production in a dose-dependent manner in response to TLR ligation.

Day 7 or Day 10 BMDCs were stimulated for 18 hours with one or no TLR ligand and different concentrations of HES ranging from 0 to 50 μ g/ml. Concentrations of cytokines in the supernatants were then measured by ELISA. Error bars represent the standard error of the mean (SEM) of triplicate wells from the same BMDCs culture. Statistics shown compare to the cytokine level of LPS-treated DCs with the production of DCs treated with LPS and 10 μ g/ml HES. Results of Student's t test: *P<0.05, **P<0.01, ***P<0.001.

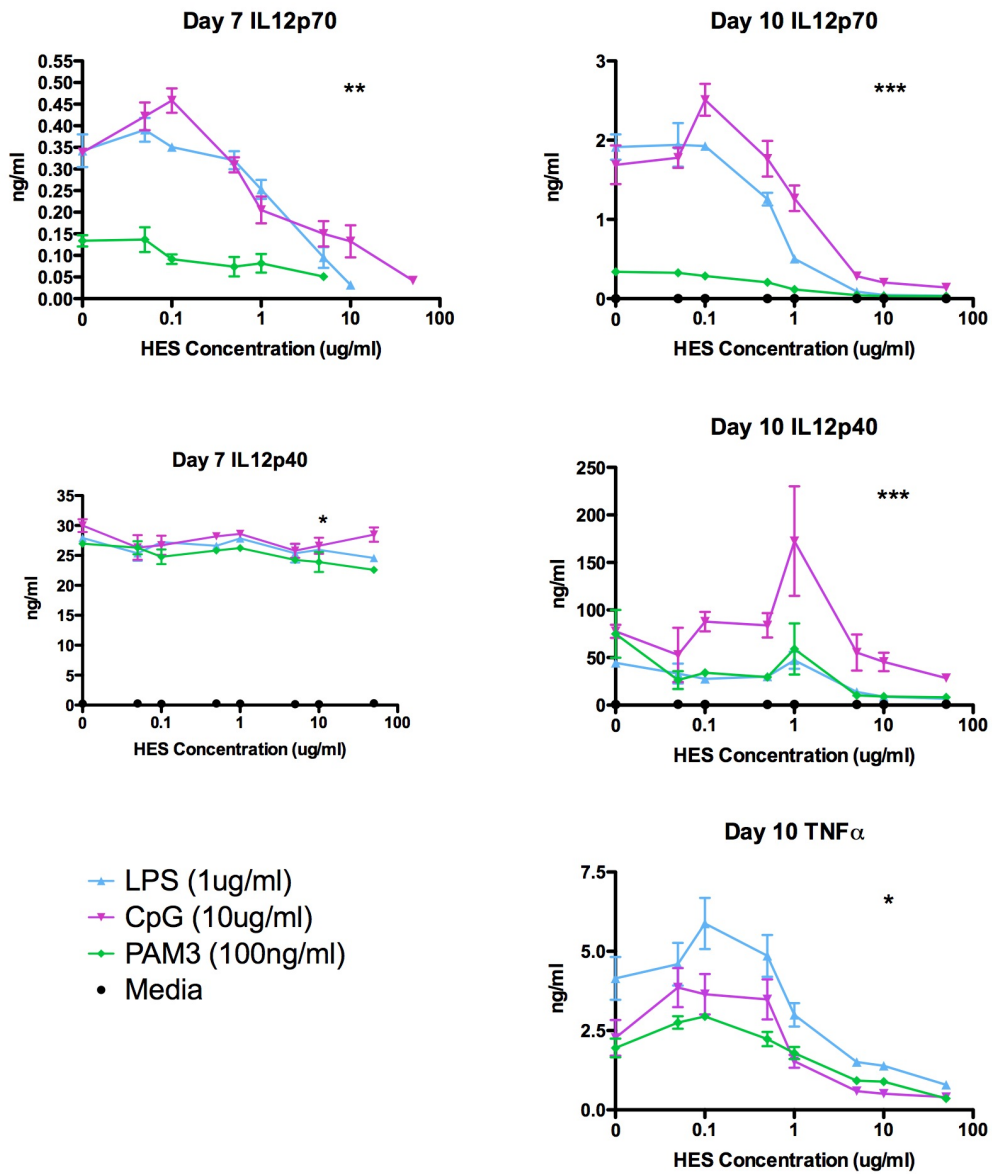


Figure 4 Part 2 - HES down-regulates IL-6, IL-12p40, IL-12p70 and TNF α production in a dose-dependent manner in response to TLR ligation.

Day 7 or Day 10 BMDCs were stimulated for 18 hours with one or no TLR ligand and different concentrations of HES ranging from 0 to 50 $\mu\text{g/ml}$. Concentrations of cytokines in the supernatants were then measured by ELISA. Error bars represent the standard error of the mean (SEM) of triplicate wells from the same BMDCs culture. Statistics shown compare to the cytokine level of LPS-treated DCs with the production of DCs treated with LPS and 10 $\mu\text{g/ml}$ HES. Results of Student's t test: *P<0.05, **P<0.01, ***P<0.001.

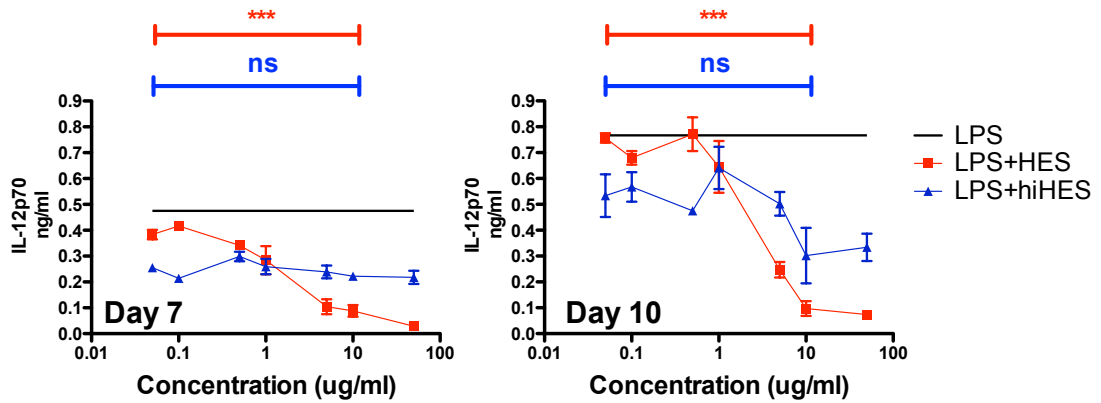


Figure 5 – Heat-inactivated HES shows a dose-independent intermediate down-regulation of IL-12p70 production.

Day 7 or Day 10 BMDCs were stimulated for 18 hours with 1 $\mu\text{g/ml}$ LPS and one concentration of HES or heat-inactivated HES (HES boiled at 95°C for 20 minutes) ranging from 0.05 to 50 $\mu\text{g/ml}$. IL-12p70 concentration in the supernatants was then measured by ELISA. The horizontal line shows the average value of triplicate wells stimulated with LPS only. Error bars represent the SEM of triplicate wells from the same BMDCs culture. Results of Student's t test: non significant (ns) $P > 0.05$, *** $P < 0.001$.

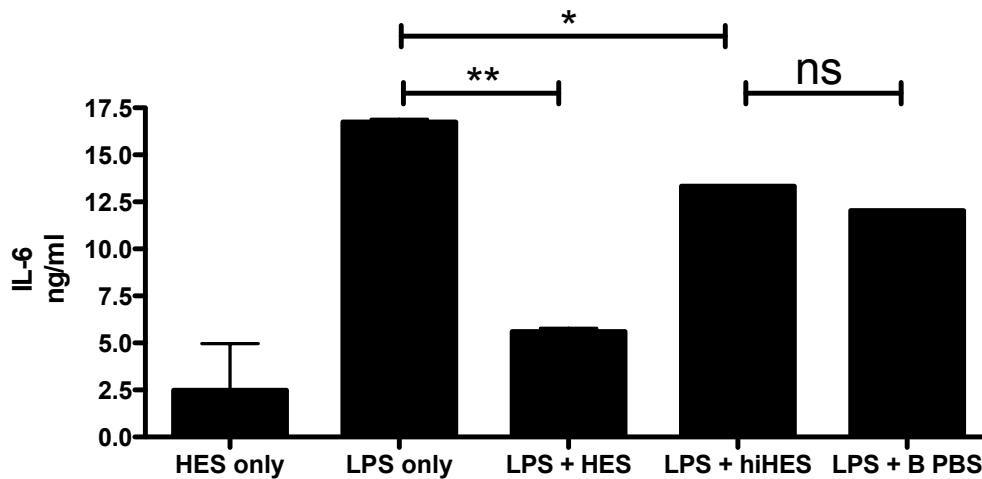


Figure 6 – The down-regulation of LPS-induced IL-6 by hiHES is similar to the effect of boiled PBS.

BMDCs were stimulated for 18 hours with a combination of PBS, boiled PBS (B PBS), 1 $\mu\text{g/ml}$ LPS, 10 $\mu\text{g/ml}$ HES or 10 $\mu\text{g/ml}$ hiHES. IL-6 and IL-12p70 concentrations in the supernatant were measured by ELISA, but there were no detectable levels of IL-12p70 in this experiment. Error bars represent the SEM of triplicate wells from the same BMDCs culture. Results of Student's t test: non significant (ns) $P > 0.05$, * $P < 0.05$, ** $P < 0.01$.

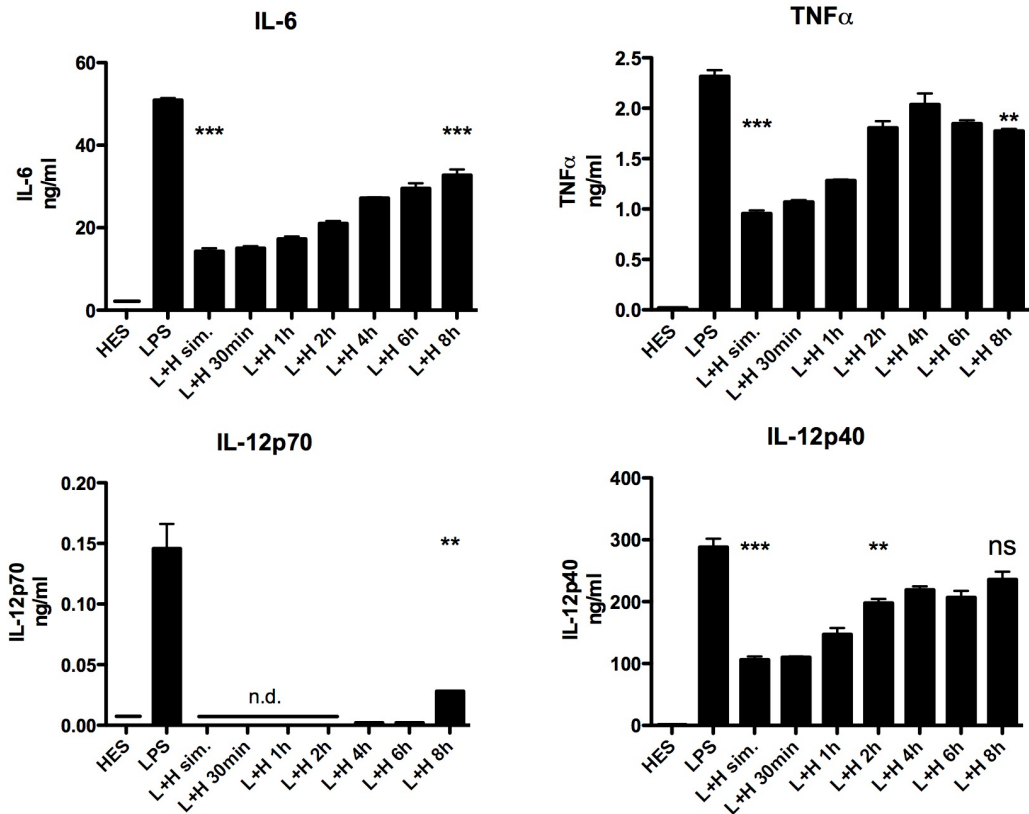


Figure 7 - HES is still able to down-regulate inflammatory cytokines up to 8 hours after TLR4 ligation.

BMDCs were stimulated either with 10 μ g/ml HES or with 1 μ g/ml LPS. After 30 minutes, 1 hour, 2,4,6 or 8 hours, 10 μ g/ml HES were added to LPS-stimulated DCs. Cytokines levels were measured by ELISA in the supernatants after 18 hours of incubation. Error bars represent the SEM of triplicate wells from the same BMDCs culture. The horizontal bars indicate that the cytokine concentration was below the detection limit. L stands for LPS, H for HES, sim. for simultaneously, n.d. for not detected. Statistics shown compare the cytokine levels of the corresponding group with the LPS-treated group. Results of Student's t test: non significant (ns) $P > 0.05$, ** $P < 0.01$, *** $P < 0.001$. Data are representative of two similar experiments.

3.2 HES can skew DCs responses independently of the TGF β pathway

Following Grainger's discovery of the TGF β -like activity of HES in his experiments on CD4⁺ T cells [39], we decided to test if this pathway was also relevant for the observed effects on DCs. First, we checked that the HES was not contaminated with some residual mammalian TGF β from the murine host. BMDCs were co-treated with LPS and neat HES or HES pre-treated for 30 minutes with a pan-vertebrate anti-TGF β antibody. No significant differences in IL-6 production, but a slight up-regulation of IL-12p70 were seen between the two groups (Fig. 8) arguing against the contamination hypothesis. The efficacy of the depletion was assessed by treating DCs with LPS and a physiological concentration of 2ng/ml recombinant human TGF β (rhTGF β) in the presence or absence of the anti-vertebrate TGF β antibody. This revealed that rhTGF β is not able to limit the cytokine response to LPS. The addition of the anti-TGF β antibody further increased the cytokine production, suggesting that the TGF β depletion was efficient.

Knowing that there was no contamination of HES with TGF β from an external source, but aware that rhTGF β had an effect on DCs cytokine production, we further assessed the immunomodulatory properties of the rhTGF β . Although DCs co-treated with LPS and TGF β showed lower levels of inflammatory cytokines than the cells treated with LPS only, this down-regulation was not dose-dependent and DCs pre-treated for 30 minutes with the ALK5 inhibitor SB431542 produced similar levels of cytokine upon LPS/rhTGF β co-stimulation (Fig. 9). These results prove that the activation of the TGF β receptor on DCs is not sufficient to achieve the strong immunomodulation showed by HES. They also suggest that the TGF β -like activity of HES discovered by John Grainger is unlikely to be part of the mechanism by which HES modifies the behaviour of DCs.

To confirm this, we compared the levels of cytokines produced upon LPS only or LPS and HES stimulation on BMDCs pre-treated for 30 minutes with DMSO or the ALK5 inhibitor SB431542. Again, the two groups showed the same ability to down-regulate the LPS-induced cytokine production (Fig. 10). Together these results suggest that the HES immunomodulation of GM-CSF-grown BMDCs is independent of the TGF β pathway. However, the possibility remains that the ALK5 inhibition was not successful and our statement should therefore be confirmed with an experiment on TGF β receptor-deficient DCs.

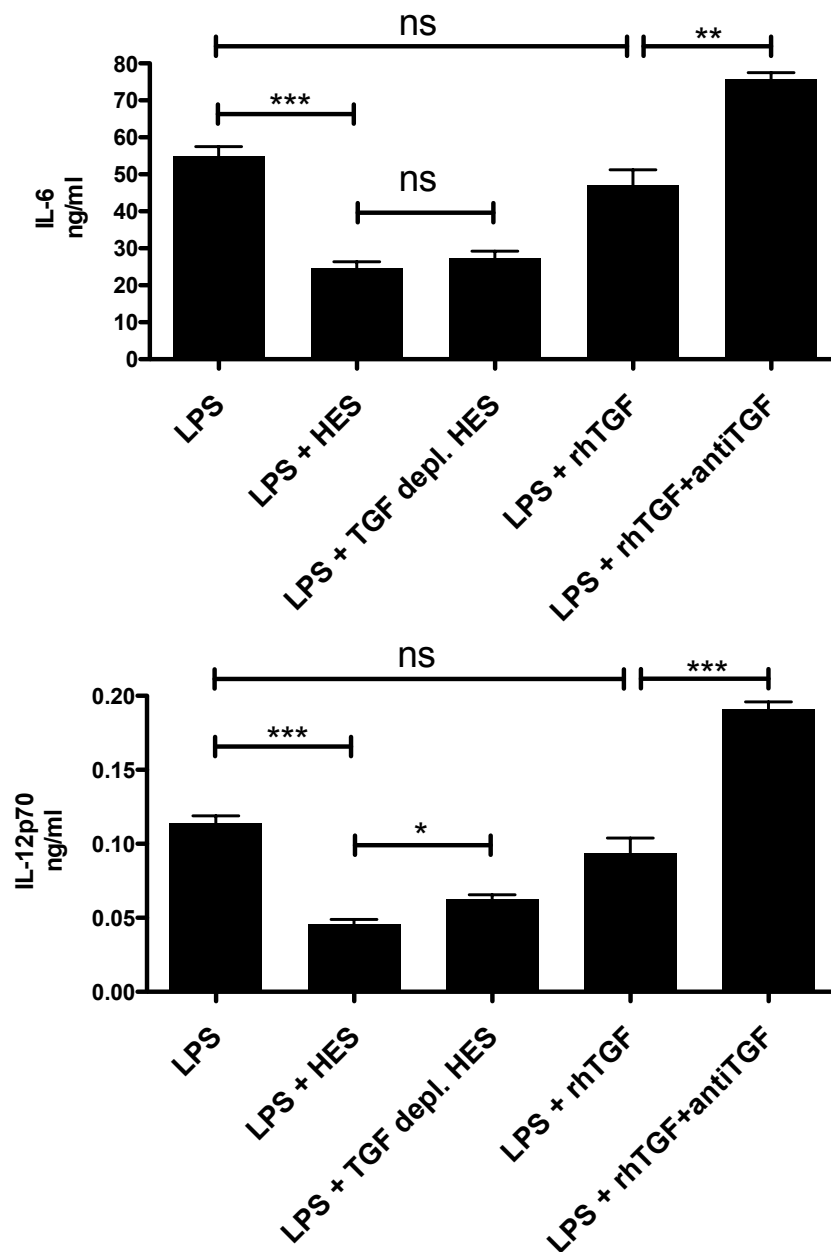


Figure 8 – HES anti-inflammatory properties are not due to a contamination with mammalian TGF β .

Some HES was incubated for 30 minutes with 10 μ g/ml of a pan-vertebrate anti-TGF β antibody to deplete it from any possible murine TGF β . DCs were stimulated for 18 hours with, 1 μ g/ml LPS, or a combination of LPS and 10 μ g/ml neat HES, TGF β -depleted HES or 2ng/ml recombinant human TGF β (rhTGF β) in presence or absence of anti-TGF β antibody. Cytokines levels in the supernatants were measured by ELISA. Error bars represent the SEM of triplicate wells from the same BMDCs culture. Results of Student's t test: non significant (ns) P>0.05, *P<0.05, **P<0.01, ***P<0.001. Data are representative of two similar experiments.

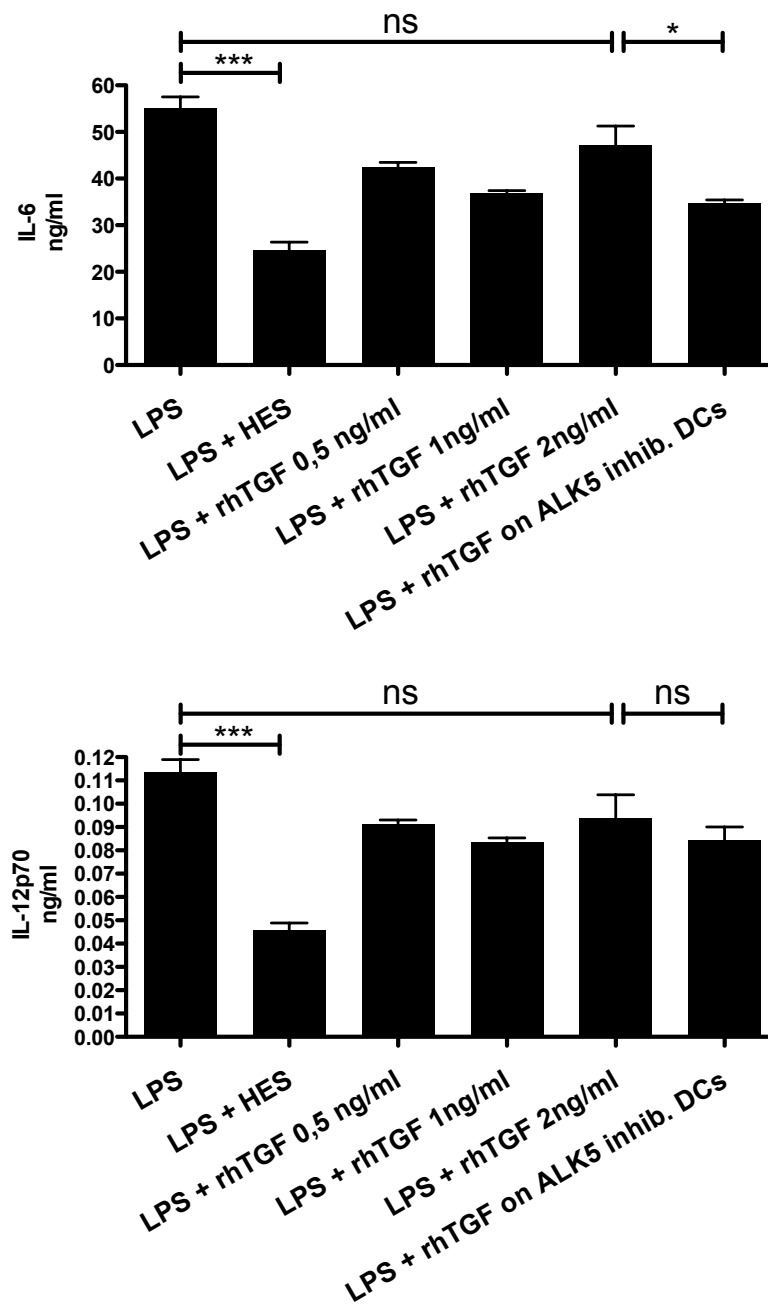


Figure 9 – The anti-inflammatory effect of recombinant human TGFβ on DCs is not dose-dependent and does not rely on the TGFβ receptor.

BMDCs were stimulated with 1μg/ml LPS and either 10μg/ml HES or different physiological concentrations of rhTGFβ (0.5ng/ml, 1ng/ml, 2ng/ml). Some BMDCs were incubated for 30 minutes with 5μM SB431542 beforehand to block the dimerisation of the TGFβ receptor and then stimulated with 1μg/ml LPS and 2ng/ml rhTGFβ. After 18 hours the supernatant were collected and the cytokines levels were measured by ELISA. Error bars represent the SEM of triplicate wells from the same BMDCs culture. Results of Student's t test: non significant (ns) P>0.05, *P<0.05, ***P<0.001. Data are representative of two similar experiments.

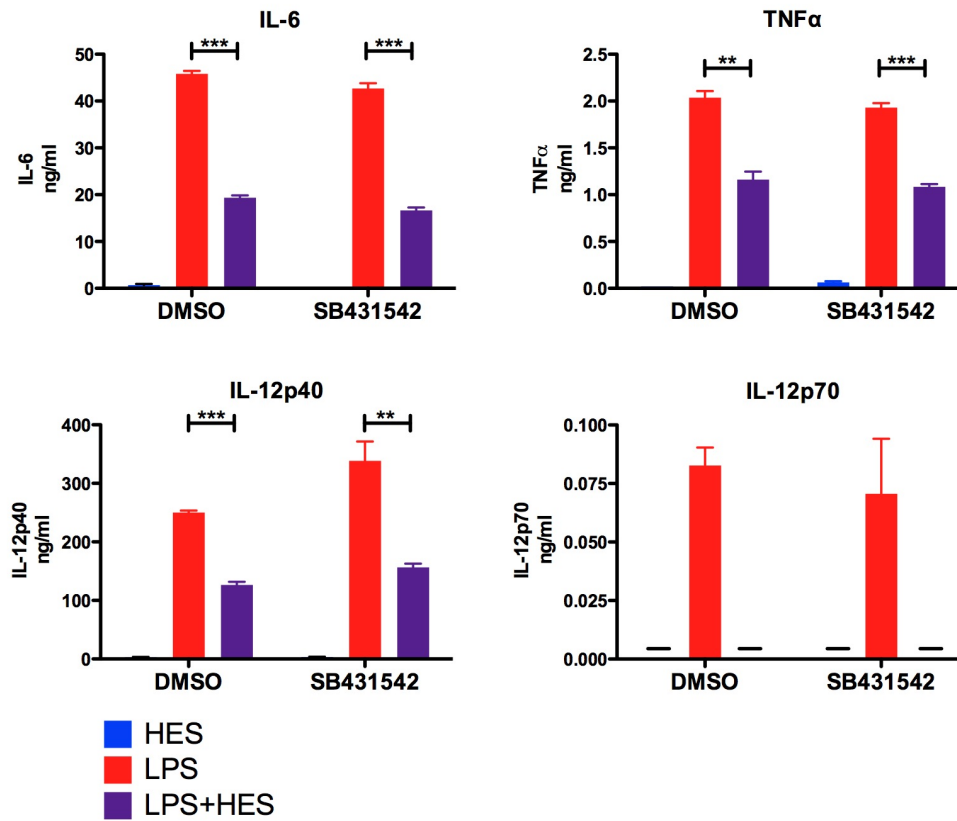


Figure 10 - HES immunomodulation of DCs is independent of the TGF β receptor.

BMDCs were incubated for 30 min with 5 μ M SB431542 or the equivalent volume of DMSO. Then, they were stimulated for 18 hours with 10 μ g/ml HES, 1 μ g/ml LPS or both. Cytokines levels in the supernatants were measured by ELISA. Error bars represent the SEM of triplicate wells from the same BMDCs culture. The horizontal bars indicate that the cytokine concentration was below the detection limit. Results of Student's t test: **P<0.01, ***P<0.001. Data are representative of two similar experiments.

3.3 HES in vitro immunomodulation of DCs is independent of Dectin-1, Dectin-2, and the Spleen tyrosine kinase pathway

Amongst all the C-type lectin receptors expressed by DCs (listed in this review [44]), we decided to assess the role of Dectin-1 and Dectin-2. Dectin-1 was targeted following work going on in Andrew MacDonald's laboratory in our institute, showing that this receptor was up-regulated on DCs following stimulation with *Schistosoma* egg antigen (SEA), another helminth product. This receptor was also shown to modulate the DC cytokine production in response to fungal PAMPs [45]. First, we checked that our DCs were expressing Dectin-1 by flow cytometry (data not shown). Then, since a blocking antibody was available in the lab, we performed a preliminary experiment and pre-treated DCs with 3µg/ml monoclonal anti-Dectin-1 antibody for 30 minutes and we compared the ability of HES to down-regulate LPS-induced cytokines on untreated DCs versus Dectin-1 blocked DCs. No difference could be spotted (Fig. 11). As the blocking efficiency of the antibody was difficult to check and thanks to the Gordon Brown laboratory in Aberdeen, we were able to perform one immunomodulation assay on Dectin-1^{-/-} BMDCs. As suggested by the preliminary experiment, HES had the same effect on the Dectin-1^{-/-} cells and their wild type counter-part (SV 129 strain). Unfortunately, IL-12p70 was not measurable in this experiment, but the perfect matching of the two IL-6 titration curves (Fig. 12) confirmed the results of the Dectin-1 blocking experiment and proved that HES does not require this receptor to modulate DC activation.

The second CLR, we were interested in was Dectin-2. This receptor had already been shown to bind SEA and to mediate the activation of the Nlrp3 inflammasome pathway upon ligation of this helminth product [31]. Unfortunately, in this case we were unable to obtain knockout mice. Therefore, we could only try to block Dectin-2 with an antibody. In a first experiment, we treated BMDCs for 30 minutes with 2, 5 or 10µg/ml anti-Dectin-2 antibody already described to be efficient at 10µg/ml [46]. Then, we treated the DCs with LPS, HES or both. DCs treated with the blocking antibody displayed similar inflammatory cytokines levels to the untreated cells with three exceptions. IL-12p70 was not detectable for the 2 and 5µg/ml anti-Dectin-2 treated groups, but the levels were so close to the detection limit that this results is possibly an artefact. The other exception is the 10µg/ml anti-Dectin-2 treated group, which showed a partial recovery of the TNFα level (Fig. 13A). To assess if this effect was real, we repeated the experiment with higher antibody levels (Fig. 13B). This second experiment showed that even at 50µg/ml the monoclonal anti-Dectin-2

antibody was unable to reverse HES effect. At 100 μ g/ml, the cytokine down-regulation was even enhanced, but at this concentration we cannot exclude that the antibody affected other molecules on the DCs via non-specific interactions. Thus, assuming that the blockage was efficient, HES does not interact with DCs through Dectin-2.

Finally, to assess more broadly the role of CLR in the HES immunomodulation of DCs, but also to confirm the Dectin-2 result, which depends on the efficiency of the blocking antibody, we blocked the downstream signal transducer Syk. Two different chemical inhibitors were used. ER-27319 was reported to block Syk binding to phosphorylated Fc γ RI ITAMs [47] and should therefore block the signalling through CLR that interact with this chain, like Dectin-2 or Mincle. BAY 61-3606 acts more downstream by blocking the Syk-mediated phosphorylation of the inhibitor of κ B ($I\kappa$ B) and therefore the translocation of NF- κ B into the nucleus and the subsequent cytokine production [48, 49] (See Appendix Fig. S 9). This inhibitor should therefore block any Syk-mediated activation of the cell.

First, we checked that the inhibitors were acting as expected. We stimulated BMDCs with hot alkali-treated zymosan that binds Dectin-1, but not its other usual receptor TLR2. This allowed us to bypass the TLR pathway and specifically study the Syk pathway. As expected, ER-27319 was unable to limit IL-6, IL-10 and TNF α production upon zymosan stimulation except for the highest dose for which we cannot exclude non-specific effects. This is logical, as Dectin-1 does not signal through the Fc γ RI chain. On the other hand, BAY 61-3606 inhibited those cytokines in a dose-dependent manner (Fig. 14). Once again IL-12p70 was below the detection threshold.

Then, we compared the ability of HES to down-regulate the LPS-induced cytokine production of DMSO-, ER-27319- or BAY 61-3606-treated BMDCs (Fig. 15). The results of this experiment are quite unique in this project, as the expression of IL-6, IL-12p70 and TNF α were most of the time highly correlated. This is not the case here and we can see some cytokine specific effects. For example, BAY 61-3606 reduces IL-6 and IL-12p70 in response to LPS indicating that this inhibitor may also partially block Syk-independent $I\kappa$ B phosphorylation or maybe that Syk also participates in the NF- κ B activation process upon LPS ligation. Surprisingly, TNF α shows the exact opposite effect. Another striking effect is the huge up-regulation of the IL-12p70 response to LPS by the ER-27319 treatment. This could suggest that a negative feedback mechanism responsible for the regulation of IL-12p70 levels is

dependent on the Fc γ RI ITAM phosphorylation. All together these results reveal an intrinsic problem of this experimental setup based on two different signalling pathways. We do not know, for example if LPS interacts with CLRs, as the results on figure 11 could suggest it, or if other receptors are interacting with the Fc γ RI chain in response to LPS. The number of variables was certainly too high to allow a clear understanding of the outcome. Thus, it could be a better idea to assess Syk implication, by looking at its phosphorylation state upon HES treatment in a future work.

Nevertheless, the effect we were looking for, that is a recovery of the cytokine production upon LPS/HES co-treatment is totally absent, suggesting, along with the Dectin-1 and Dectin-2 experiments, that HES does not need the Syk pathway to modulate the DCs. However, this statement needs further studies to be confirmed.

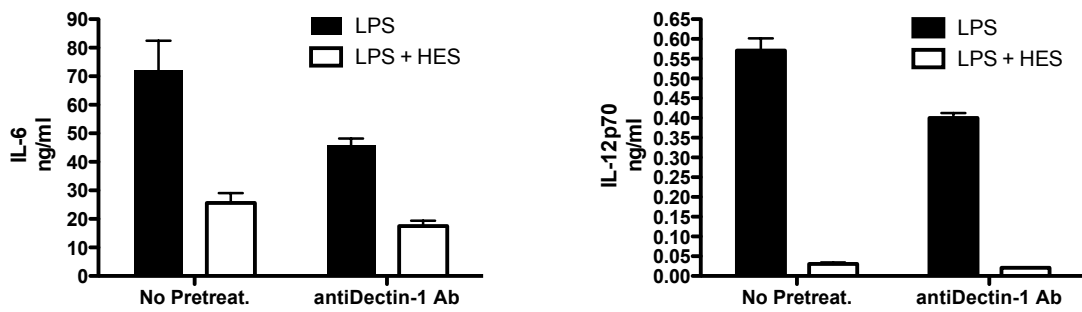


Figure 11 - HES can still modulate the cytokine response to LPS after antibody blocking of Dectin-1.

BMDCs were incubated 30 minutes with or without 3 μ g/ml monoclonal anti-Dectin-1 antibody and then stimulated for 18 hours with 1 μ g/ml LPS in presence or absence of 1 μ g/ml HES. Cytokines levels in the supernatants were measured by ELISA. Error bars represent the SEM of triplicate wells from the same BMDCs culture.

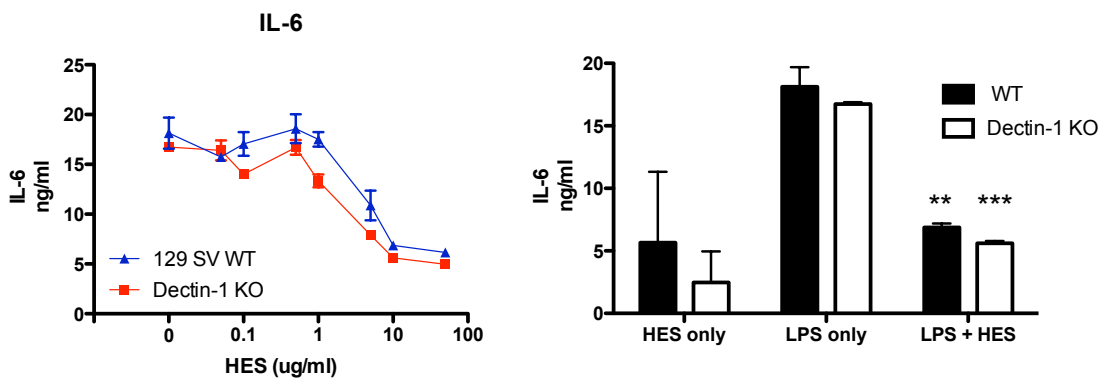


Figure 12 - Dectin-1^{-/-} BMDCs display the same HES immunomodulation pattern as the corresponding wild type DCs.

BMDCs were derived from a Dectin-1^{-/-} mouse and its corresponding wild type strain (129 SV). DCs were then stimulated with 1 μ g/ml LPS and different concentrations of HES, ranging from 0.05 to 50 μ g/ml or with only 10 μ g/ml HES. IL-6 levels were measured by ELISA in the supernatants after 18 hours incubation. In this experiment, IL-12p70 levels were below the detection limit. Error bars represent the SEM of triplicate wells from the same BMDCs culture. Statistics shown compare the LPS versus LPS+HES treatments for each strain. Results of Student's t test: **P<0.01, ***P<0.001.

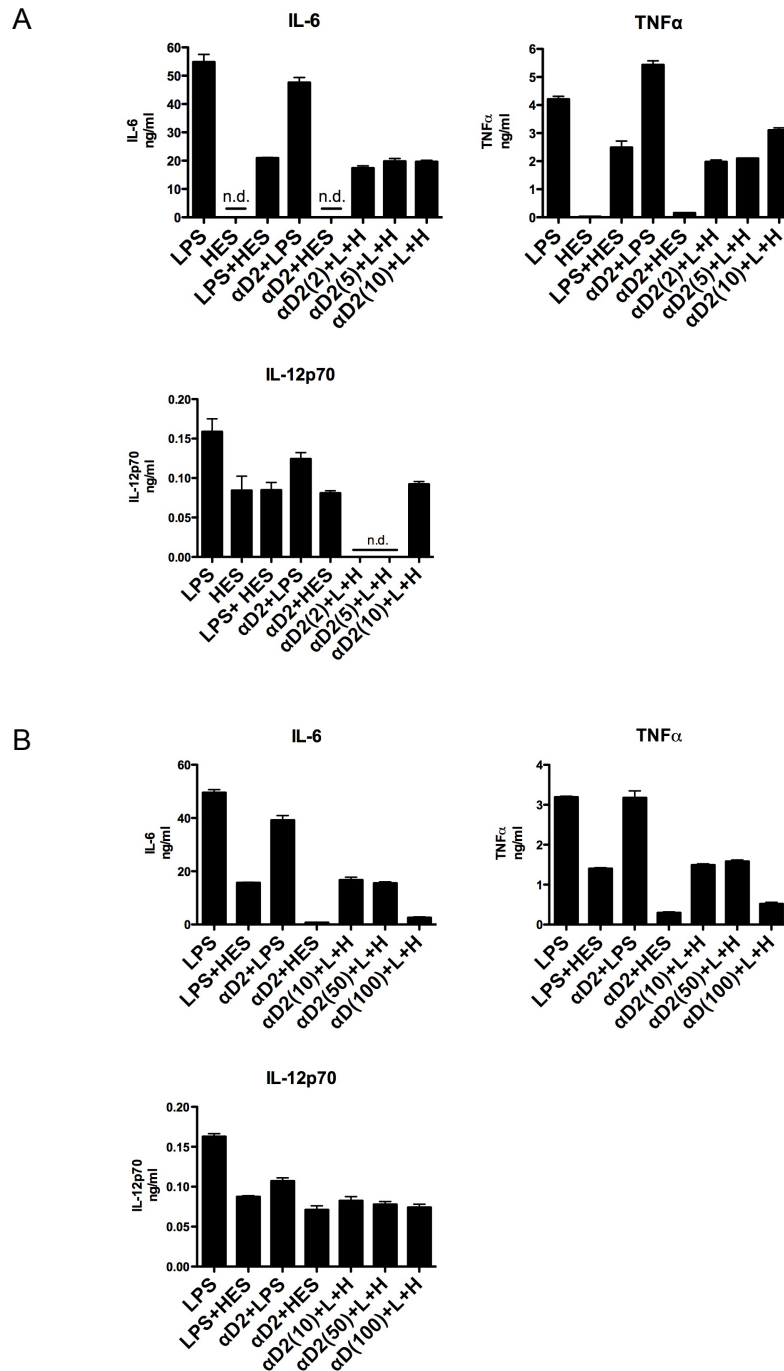


Figure 13 - Dectin-2 antibody blockage does not alter HES immunomodulatory properties.

BMDCs were pre-treated or not with different concentrations of a monoclonal anti-Dectin-2 antibody (α D2) for 30 minutes. Concentrations in brackets are in μ g/ml. Then, they were stimulated with 1μ g/ml LPS, 10μ g/ml HES or both (L+H). After 18 hours, cytokines levels in the supernatants were measured by ELISA. Error bars represent the SEM of triplicate wells from the same BMDCs culture. The horizontal bars indicate that the cytokine concentration was below the detection limit (n.d. stands for not detected). Panels A and B present two different experiments.

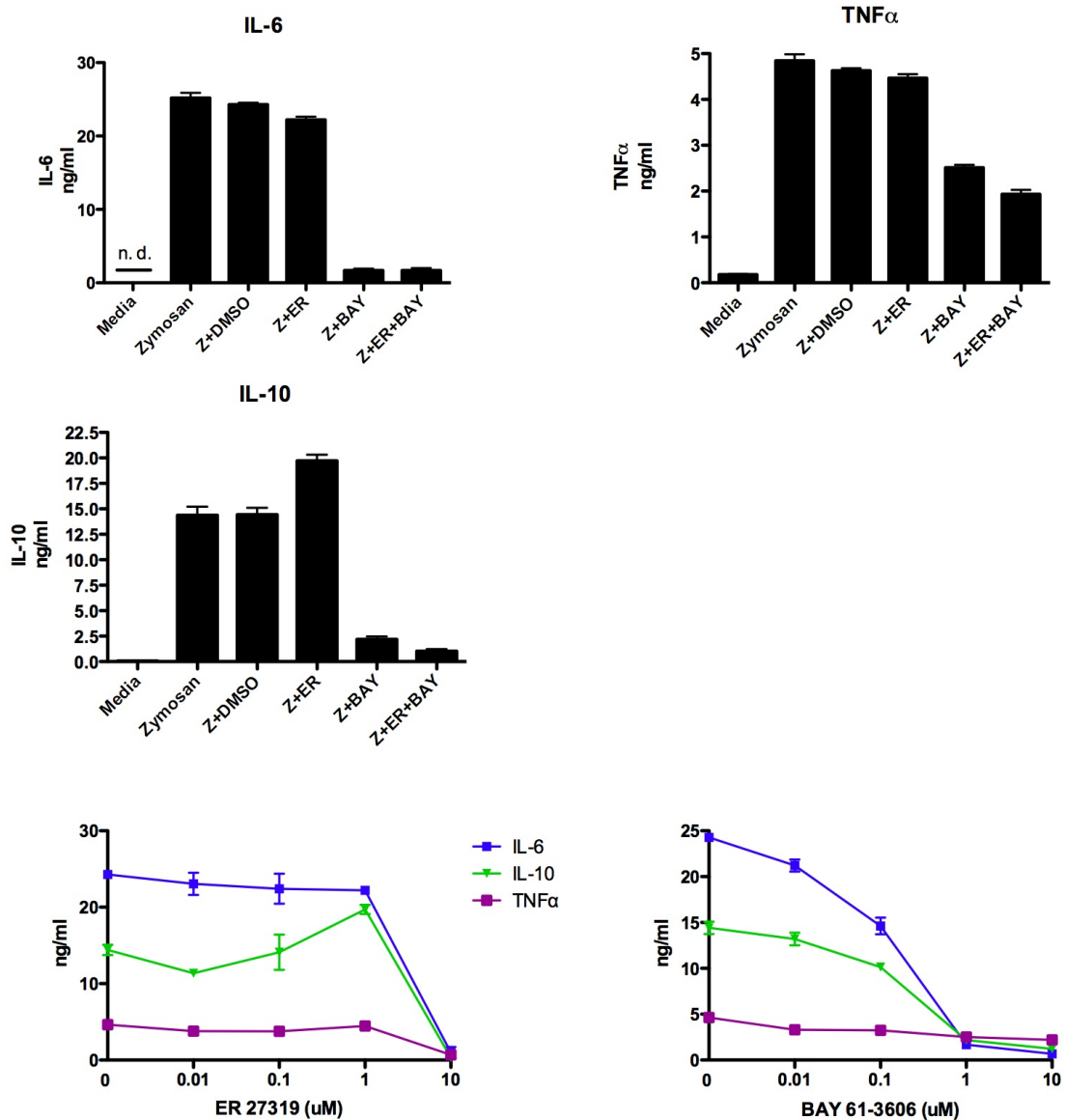


Figure 14 - The Syk inhibitor BAY 61-3606, but not ER-27319 is able to block cytokine production upon Dectin-1 ligation.

BMDCs were pre-treated for 30 minutes with different doses ER-27319, BAY 61-3606 or an equivalent volume of DMSO. DCs were then stimulated with 100 μ g/ml hot alkali treated zymosan (Z) that specifically binds to Dectin-1 but not TLR2. After 18 hours, cytokines levels in the supernatants were measured by ELISA. Error bars represent the SEM of triplicate wells from the same BMDCs culture. The horizontal bars indicate that the cytokine concentration was below the detection limit (n.d. stands for not detected).

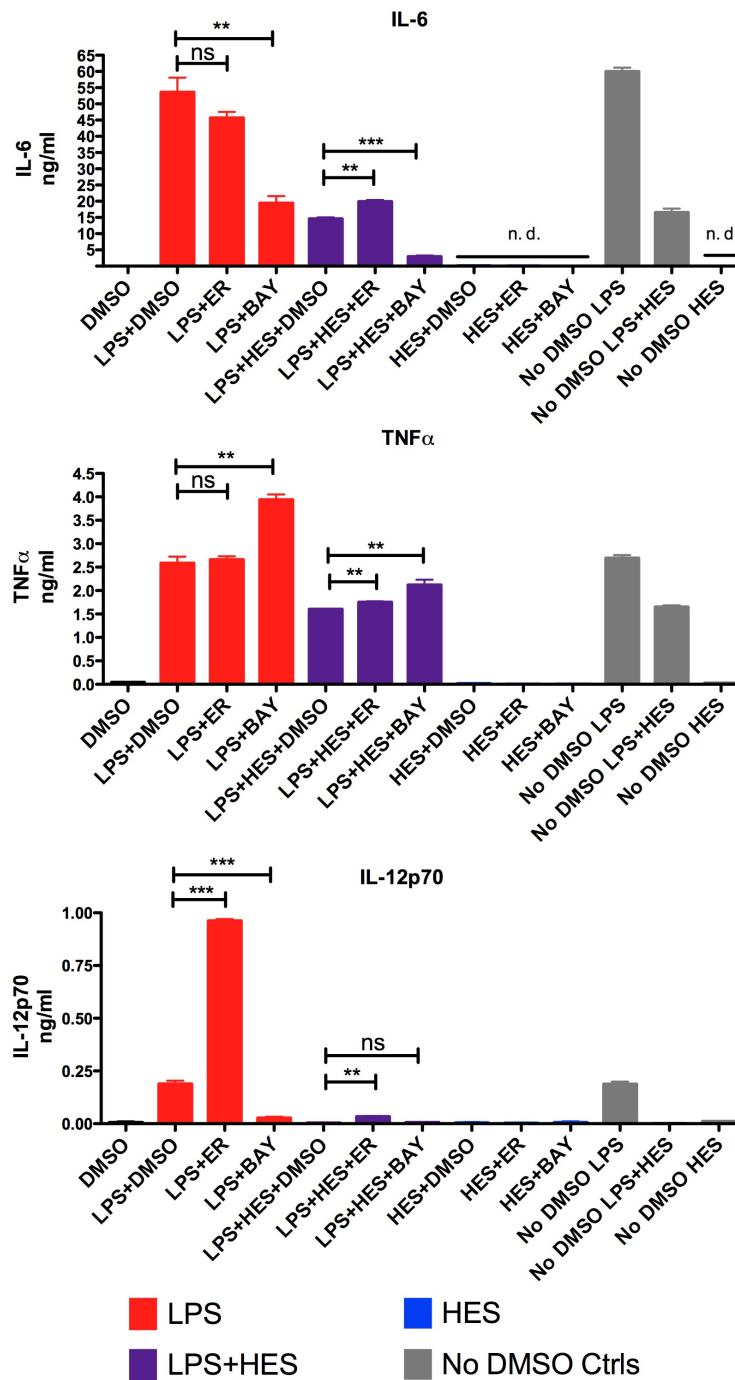


Figure 15 – Syk inhibitors are unable to restore inflammatory cytokines levels upon LPS/HES co-stimulation.

BMDCs were incubated for 30 minutes with 1 μ M ER-27319, 1 μ M BAY 61-3606, an equivalent volume of DMSO or not treated at all to check for any DMSO induced effect. DCs were then stimulated with 1 μ g/ml LPS, 10 μ g/ml HES or both. After 18 hours, cytokines levels in the supernatants were measured by ELISA. Error bars represent the SEM of triplicate wells from the same BMDCs culture. The horizontal bars indicate that the cytokine concentration was below the detection limit (n.d. stands for not detected). Results of Student's t test: non significant (ns) $P > 0.05$, ** $P < 0.01$, *** $P < 0.001$.

3.4 HES in vitro immunomodulation of DCs is independent of the TLR and PI3K pathways

Although Segura et al. [37] showed that HES is still able to down-regulate IL-12p70 production of TLR2^{-/-} and TLR4^{-/-} DCs, we decided to further study the pathway by measuring the cytokine response of MyD88 and TRIF deficient DCs. This allows assessing the role of all TLR ligands in a few experiments, as all of them are dependent on one or both of these adaptors to transmit the ligation signal. Moreover, even though retracted, the recent discovery by Puneet et al. [25] that the anti-inflammatory effects of ES-62, were due to its ability to induce the autophagosomal degradation of MyD88 suggest that these adapter molecules could be crucial targets for HES. In addition, based on a few papers [21, 23, 24, 26] suggesting that PI3K plays a regulatory role in the response to TLR ligation, we also investigated the response of PI3K-inhibited BMDCs to LPS and HES. This study of a lower level of signalling is also interesting since the PI3K/Akt/mTOR pathway was suggested to modulate both MAPK and NF- κ B pathways in human monocytes [50].

In the first place, we treated BMDCs for 30 minutes with different concentrations of wortmannin, a common PI3K inhibitor. These DCs were then stimulated with LPS in the presence or absence of HES. At low and medium dose, wortmannin induced the up-regulation of IL-12p40 and p70 in response to LPS. On the contrary, at a high dose, PI3K inhibition led to a diminution of all measured cytokines, but TNF α (Fig. 16). This could seem to contradict the previously cited papers, but, even though IL-12 levels were lower when the cells were pre-treated with 1 μ M wortmannin rather than 10 or 100nM, they were still higher than the DMSO-treated DCs. Moreover, the increase of the wortmannin dose also tended to inhibit the IL-10 production, supporting the idea that the PI3K pathway plays an anti-inflammatory role in the setting of BMDCs activation by TLR ligation. Unfortunately, this effect turned out to be non significant. When looking at the LPS and HES co-treated group, we can see that, with the exception of TNF α , the wortmannin treatment does not induce a recovery of the inflammatory cytokine levels. TNF α levels were increased upon 100nM or 1 μ M wortmannin pre-treatment, but they stayed below the corresponding values of the LPS-treated group. So, these results suggest that the HES immunomodulation of DCs is independent of the PI3K pathway.

Second, we wanted to study the role of MyD88 and TRIF. To this end, we stimulated DCs derived from MyD88^{-/-}, TRIF^{-/-} or wild type bone marrow with four different TLR ligands and different concentrations of HES. As TLR4 can signal through both

MyD88- and TRIF-dependent pathways, the stimulation with LPS allows measuring the immunomodulatory effects of HES in the absence of one of these molecules, as the other intact pathway will still induce some cytokine production. Stimulation with CpG, Pam3 and R848 were used to assess the reproducibility of the results on a broader range of TLRs and check that the MyD88-dependent TLR9, TLR1/2 and TLR7/8 were unable to induce an inflammatory response in the MyD88 deficient cell lines. Their response was expected to be unaltered in TRIF^{-/-} mice.

As expected, MyD88^{-/-} DCs did not produce any IL-6 upon CpG, Pam3 or R848 stimulation (Fig. 17). Very low levels of this cytokine were still detectable after LPS activation showing that MyD88 is crucial for IL-6 production upon TLR4 ligation, even if the TRIF pathway was still functional. The TNF α levels were less affected although MyD88^{-/-} DCs produced about four times less TNF α than wild type DCs upon LPS ligation. When stimulated with the other TLR ligands, the TNF α production by MyD88^{-/-} DCs was similar to the levels induced by the non-stimulated control. CpG and Pam3 stimulation induced similar levels of IL-6 and TNF α in both wild type and TRIF^{-/-} groups, but surprisingly, the production of these cytokines was diminished in TRIF^{-/-} upon R848 stimulation suggesting a potential role for TRIF in the response to R848. More logically, the IL-6 levels reduction was strong upon LPS ligation as TLR4 also signals through the TRIF pathway.

More importantly, every condition displayed the same dose-dependent cytokine down-regulation in response to HES co-treatment. Even LPS induced IL-6 produced by MyD88^{-/-} BMDCs was reduced from 2.3ng/ml without HES to 1ng/ml with 10 μ g/ml HES. These results provide strong evidence that the HES modulation of DCs is independent of the TLR pathway. Moreover, they are supported by the flow cytometry analysis of co-stimulatory markers expressed on DCs after 18 hours stimulation (See Appendix Fig. S 1 and S 2).

Nevertheless, it was still possible that HES acted redundantly through both MyD88 and TRIF pathways. Thus, to rule out this hypothesis, we decided to perform an experiment on MyD88/TRIF double knockout BMDCs. In this setup LPS is useless for cell stimulation. Therefore, we tried to activate DCs through a CD40 ligation, as this had already been described to up-regulate co-stimulatory markers and IL-12 on ex-vivo DCs [51]. First, we wanted to measure the outcome of such a ligation in our setup. Thus, we treated BMDCs with different concentrations of the FGK45 monoclonal anti-CD40 antibody and looked at their cytokine production and co-stimulatory marker up-regulation (Fig. 18). At 10 μ g/ml, the antibody was able to

induce low levels of IL-12p40 and very low levels of IL-6, but not TNF α and IL-12p70. At 50 μ g/ml, IL-6, IL-12p40 and TNF α were more strongly induced, but this is probably due to an antibody independent effect, as these results do not fit the titration curves. A low endotoxin contamination of the antibody stock could, for example, explain this result. The co-stimulatory markers, and particularly CD86, seem to be better read-outs, as they were strongly up-regulated upon CD40 ligation. CD40 fluorescence intensity was negatively correlated with anti-CD40 concentration, indicating that the binding of this antibody was efficiently competing with the fluorochrome-associated anti-CD40 antibody.

In this experiment we also assessed the ability of HES to modify the anti-CD40-induced DC activation by co-treating some anti-CD40-activated DCs with 10 μ g/ml HES. Despite the fact that IL-6 and IL-12p70 were not induced by CD40 ligation, the other read-outs showed a HES immunomodulation of anti-CD40-activated cells comparable to the modulation of LPS-induced DCs (Fig. 19). Together, these results indicate that CD40 ligation can be used to assess the effect of HES on MyD88^{-/-}xTRIF^{-/-} cells.

Thus, we treated wild type and MyD88^{-/-}xTRIF^{-/-} BMDCs with anti-CD40 and a titration of HES. Unfortunately, the anti-CD40 activation did not work as well as in the previous experiment and, since this experiment was performed just before the end of the project, we were not able to repeat it. No IL-12p40 induction was detected. Nevertheless, the down-regulation of CD80, CD86 and MHC class II by the addition of HES on activated cells can clearly be seen on both cell strains (Fig. 20). This confirms that HES does not require either MyD88 or TRIF to modulate the DCs and therefore excludes the participation of any TLR ligand in the process.

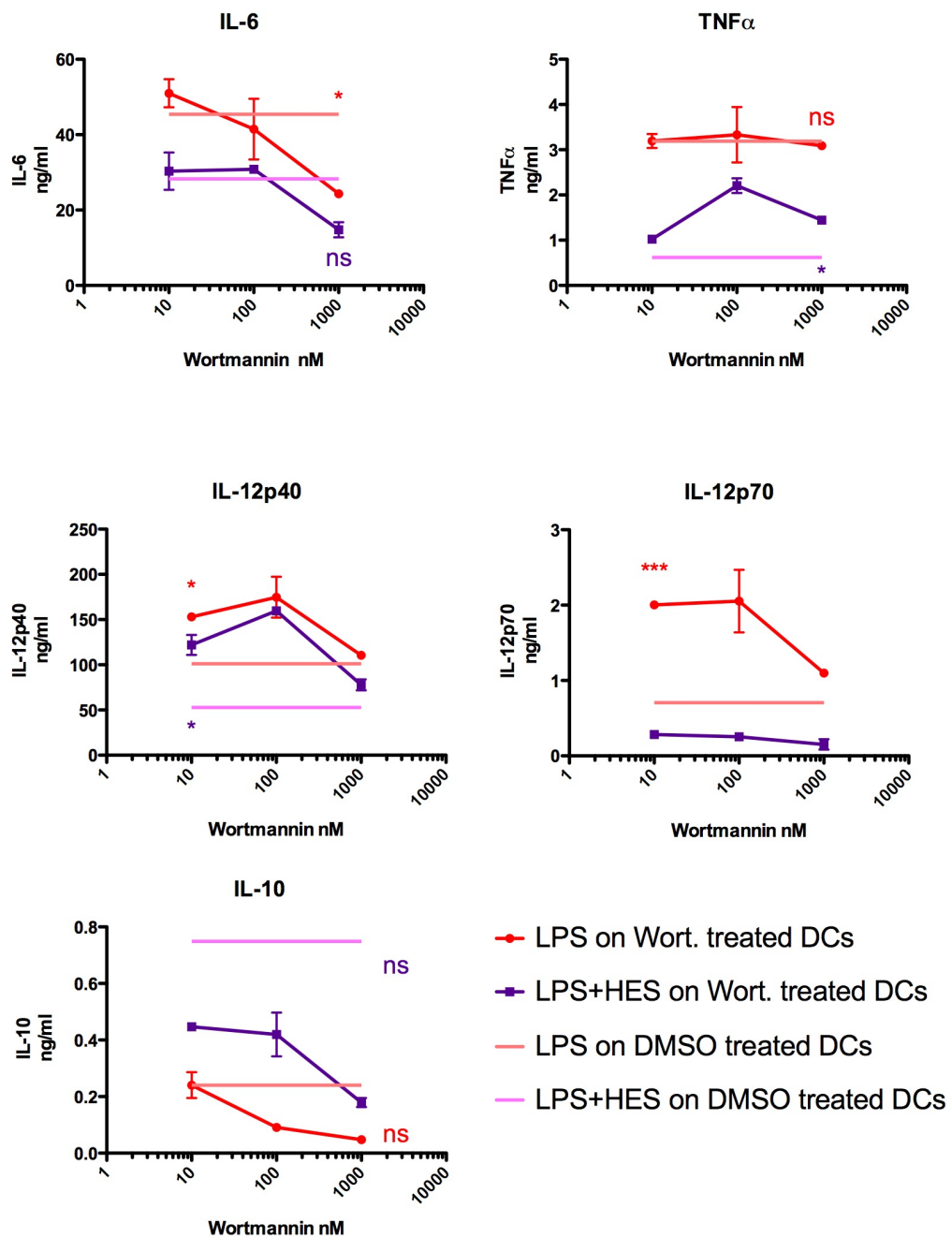


Figure 16 - The inhibition of the phosphoinositide-3-kinase does not alter the HES immunomodulation of DCs.

BMDCs were pre-treated with DMSO, 10nM, 100nM or 1 μ M wortmannin for 30 minutes before their stimulation with 1 μ g/ml LPS in presence or absence of 10 μ g/ml HES. After 18 hours incubation, cytokines levels in the supernatants were measured by ELISA. DMSO-treated DCs did not produce IL-12p70 upon LPS and HES co-treatment. Average values of triplicate wells are plotted for the DMSO-treated groups. Error bars represent the SEM of triplicate wells from the same BMDCs culture. Statistics shown compare the level of the DMSO control with one wortmannin treated group according to the position and colour of the symbol. Results of Student's t test: non significant (ns) $P > 0.05$, * $P < 0.05$, *** $P < 0.001$. Data are representative of two similar experiments.

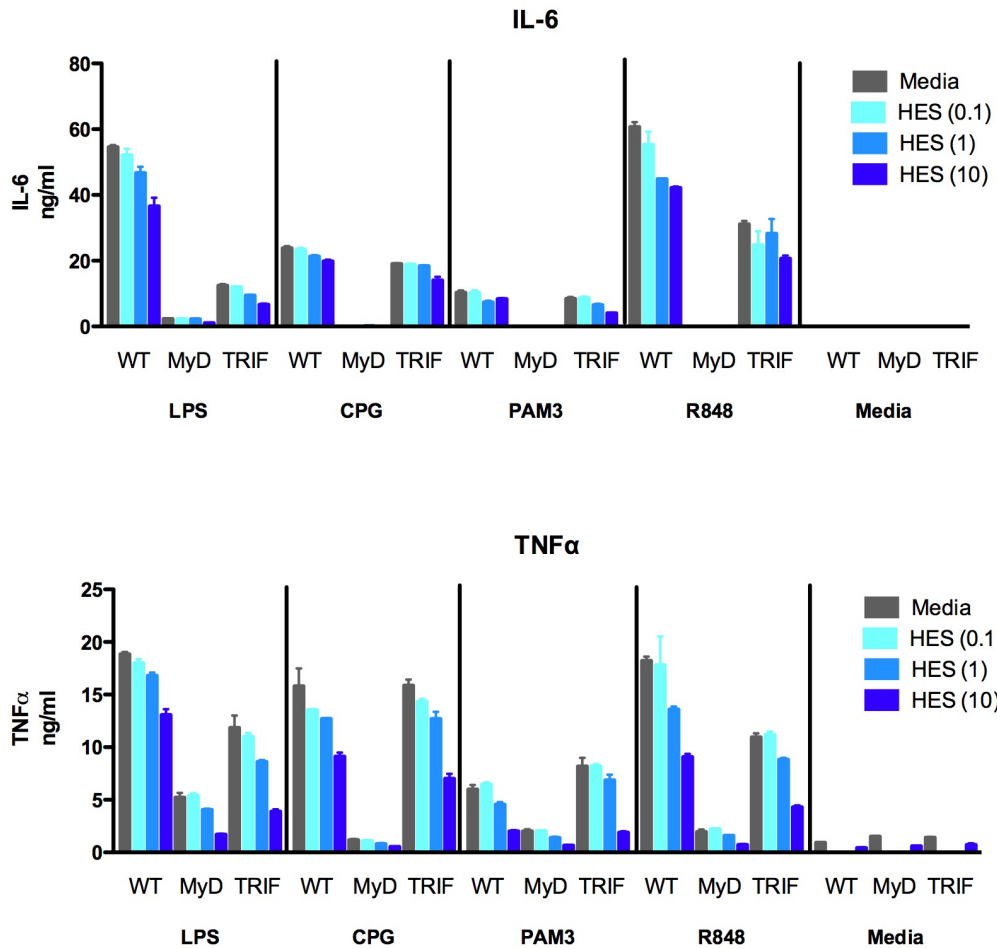


Figure 17 - HES down-regulates TLR ligation-induced IL-6 and TNF α in both MyD88^{-/-} and TRIF^{-/-} BMDCs.

Wild type, MyD88^{-/-} and TRIF^{-/-} BMDCs were stimulated for 18 hours with a combination of one TLR ligand or media and one HES concentration or media. LPS was used at 1 μ g/ml, CpG at 10 μ g/ml, Pam3 at 100ng/ml and R848 at 1 μ g/ml. HES was added at 0.1, 1 or 10 μ g/ml. Cytokines levels in the supernatants were measured by ELISA. Error bars represent the SEM of triplicate wells from the same BMDCs culture. This is representative of two similar experiments.

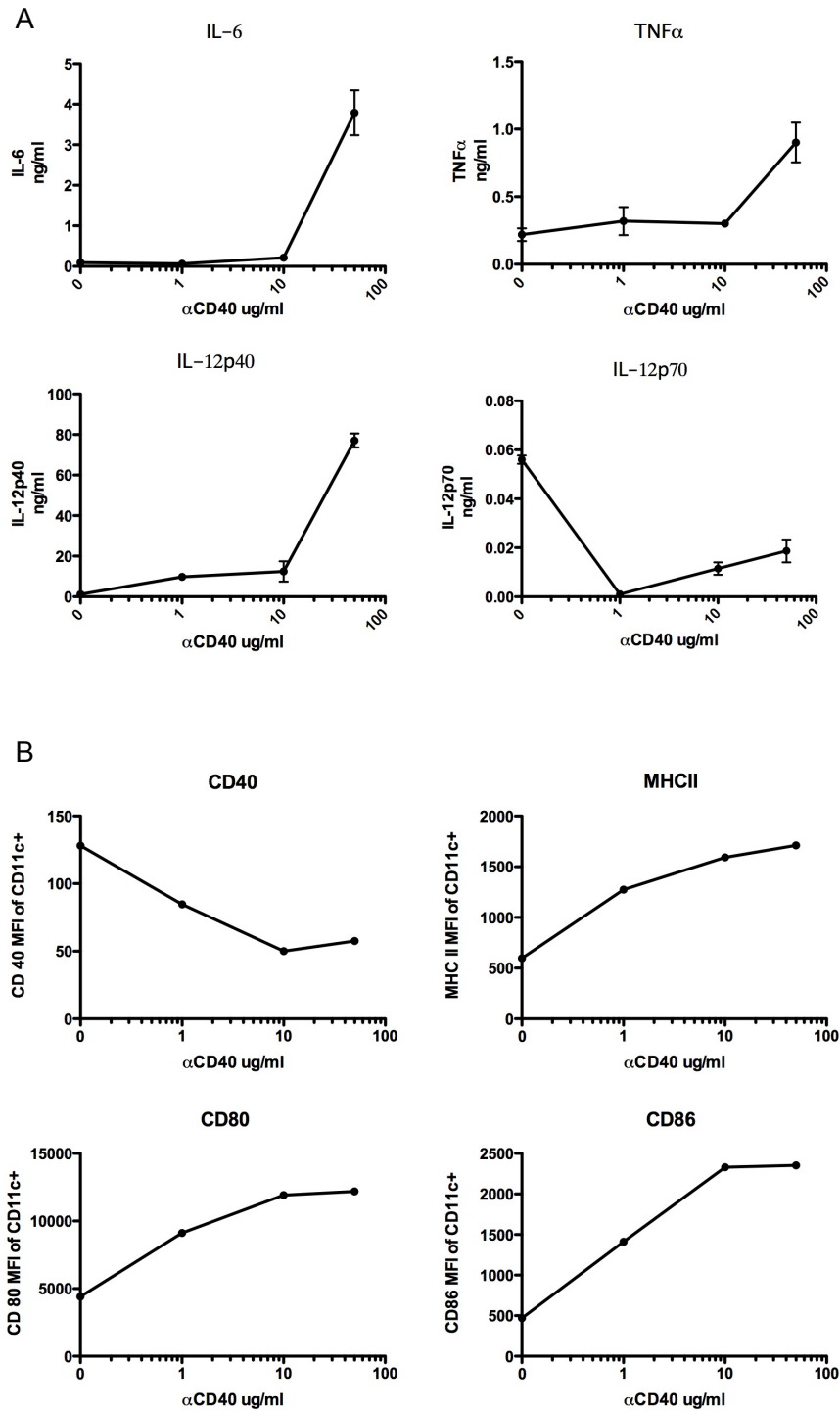


Figure 18 - CD40 ligation induces IL-12p40 production and co-stimulatory markers up-regulation by BMDCs.

BMDCs were stimulated with 1, 10 or 50 $\mu\text{g/ml}$ FGK45, an anti-CD40 antibody (αCD40), or kept in media for 18 hours. Cytokines concentrations in the supernatants were then measured by ELISA (panel A) and the DCs were stained for CD11c, MHC class II, CD40, CD80 and CD86. Geometric means of the fluorescence intensity (MFI) for CD40, CD80, CD86 and MHC class II of CD11c positive gated live cells were then plotted (panel B). Error bars represent the SEM of triplicate wells from the same BMDCs culture. Triplicates were pooled before surface staining.

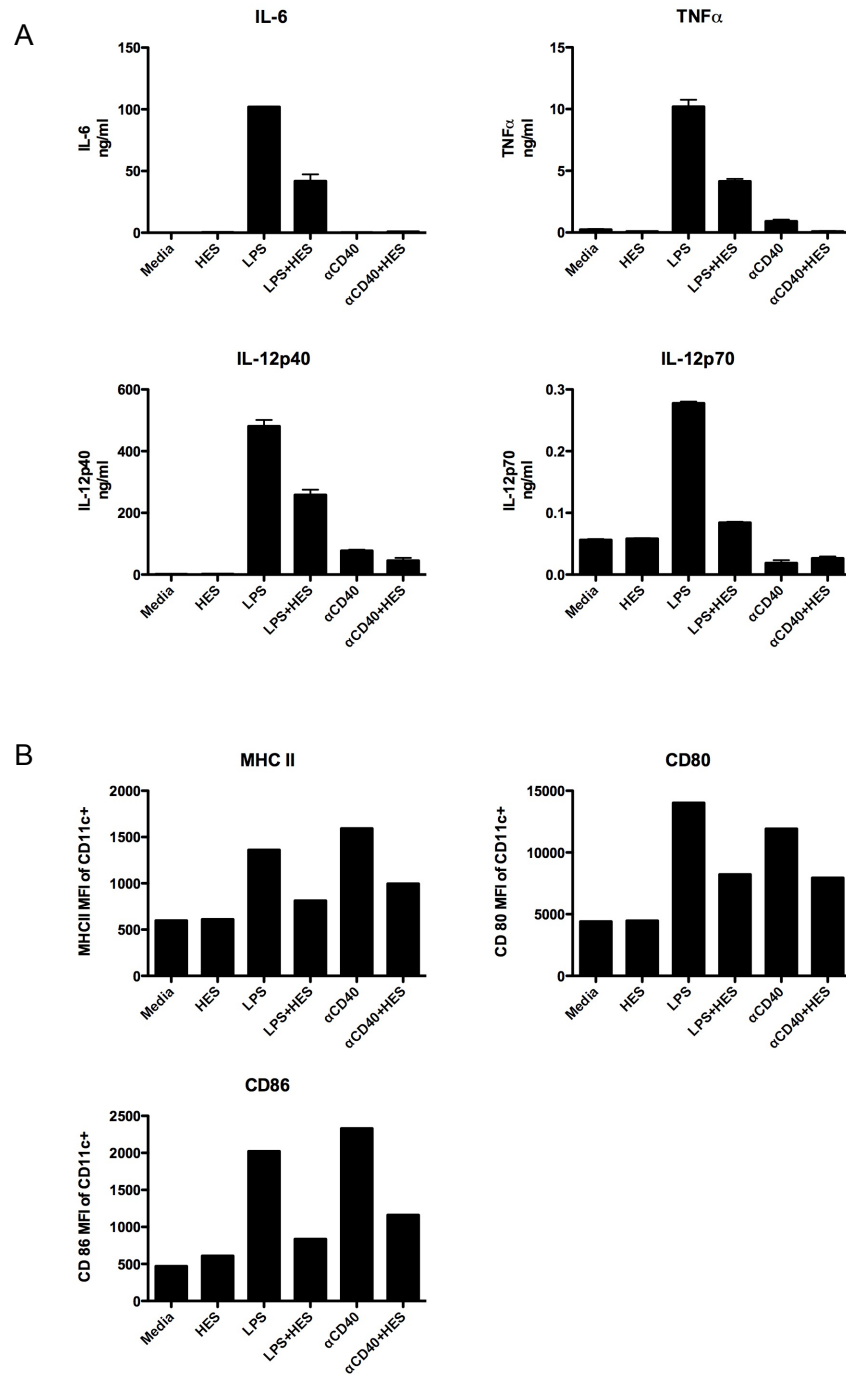


Figure 19 - HES is able to limit IL-12p40, TNF α production and co-stimulatory markers expression of anti-CD40 stimulated BMDCs.

In addition to the usual immunomodulation assay, BMDCs were also stimulated with 10 μ g/ml anti-CD40 antibody (α CD40) in presence or absence of 10 μ g/ml HES. Cytokines concentrations in the supernatants were then measured by ELISA (panel A) and the DCs were stained for CD11c, MHC class II, CD40, CD80 and CD86. Geometric means of the fluorescence intensity (MFI) for CD80, CD86 and MHC class II of CD11c positive gated cells were the plotted (panel B). Error bars represent the SEM of triplicate wells from the same BMDCs culture. Triplicates were pooled before surface staining.

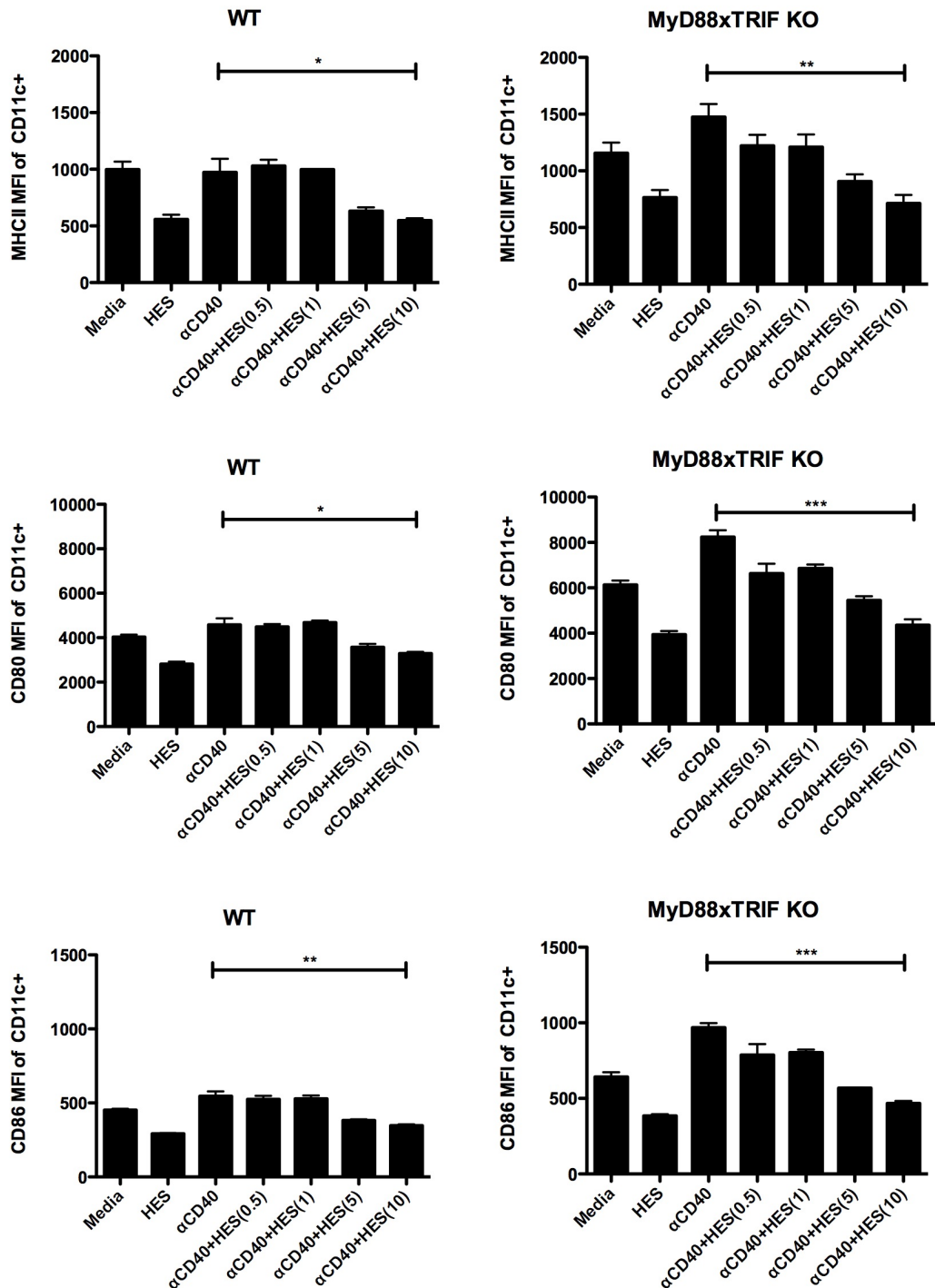


Figure 20 - HES down-regulates CD80, CD86 and MHC class II expression on activated wild type and MyD88^{-/-}xTRIF^{-/-} in a similar manner.

Wild type (WT) or MyD88xTRIF double knockout BMDCs were stimulated with 10μg/ml HES, 10μg/ml anti-CD40 antibody (αCD40) or a combination of anti-CD40 and 0.5, 1, 5, or 10 μg/ml HES. After 18 hours incubations, cells were stained for CD11c, MHC class II, CD40, CD80, CD86 and the geometric mean of the fluorescence intensity was plotted. Error bars represent the SEM of triplicate wells from the same BMDCs culture. Results of Student's t test: * P<0.05, ** P<0.01, ***P<0.001.

3.5 HES fractions analysis

In parallel with our attempts to identify the molecular pathway targeted by HES, we also tried to define which particular compounds of this complex mixture were responsible for the global effect. To this end, we compared the immunomodulatory properties of 24 HES molecular weight fractions to the effect of the whole product. These fractions, resulting from a size fractionation on a Superdex 200 column (GE Health care), were kindly provided by James Hewitson and Kara Filbey.

First, we have to mention that at least another research group performed the same kind of HES size fractionation combined with functional assays to determine the immunoactive compounds of HES. In 2006, Rzepecka et al. [40, 52] defined an active fraction of HES by looking at the ability of the fractions to inhibit the antigen-specific cellular proliferation of mesenteric lymph node cells. This allowed them to narrow down the number of molecular targets to seven. In a second paper [40], they suggested a mechanism involving interaction between *H. polygyrus* calreticulin (HpCRT) and the DC scavenger receptor-A. Following these results, Mark Pearson, a former researcher in the lab, had purified bacterially expressed recombinant HpCRT in with the hope of developing vaccines. Therefore, in order to avoid going through a similar process in our setup and end up with the same candidate, we tried to directly treat BMDCs with recombinant HpCRT and see if it had an effect.

To begin with, we had to remove the bacterial contaminants from the protein solution to avoid a direct activation of the TLR pathway that could mask anti-inflammatory effects. This was performed by the repeated incubations with Triton X114 followed by centrifugation. This reagent forms micelles that trap LPS. The centrifugations separate the solution into a protein-containing aqueous phase and a LPS-containing lipid phase. We then treated LPS-activated DCs with different concentrations of HpCRT and noticed that this protein had no immunomodulatory effects (Fig. 21). Moreover, HpCRT seemed to activate the cells to a certain extent by itself. Nevertheless, this “fishing” experiment cannot exclude a possible glycan-mediated effect of native HpCRT, as the recombinant protein was produced by bacteria and therefore lacks post-translational modifications.

Next, we conducted our own fractions analysis. We co-treated BMDCs with 1 μ g/ml LPS and 10 μ g/ml HES or the equivalent of 10 μ g/ml of each fraction as estimated from the initial amount of HES run through the size fractionation column and the ratio of the fraction volume to the total elution volume. The down-regulation of the inflammatory cytokines of each fraction was compared to the maximal

immunomodulation achieved by total HES and if this ratio reached more than 40% the fraction was highlighted on the graph (Fig. 22). This experiment was repeated on two different BMDCs cultures and, after comparison of the percentages of cytokine inhibition, we concluded that fraction 14 was the most potent in limiting IL-6, IL-12p70 and TNF α production, even though fractions 8, 9 and 10 also displayed immunomodulatory properties (Table 3).

From there, we decided to compare the protein as well as the glycan content of fraction 14 with its neighbouring fractions 13 and 15 that showed weak immunomodulatory properties with the hope of finding few differences between these size-related fractions. Regarding the proteins, we first performed 2D SDS-PAGE of these three fractions and silver stained them (pictures in Appendix, Fig. S 3 and S 4). As the patterns were very different, it was impossible to select specific spots for a targeted mass spectrometry analysis. Therefore, we considered sending the whole fraction for a global mass spectrometry analysis. However, as another fraction had already been sent for the same purpose, we decided to wait for the results of this other fraction before sending fraction 14. Unfortunately, the mass spectrometry turned out not to be as useful as expected, since more than a hundred identities were detected in the other fraction, suggesting that the size fractionation on the Superdex column was not sufficient to significantly reduce the number of molecular targets in a single fraction. For this reason, fraction 14 has not yet been analysed by mass spectrometry. Ion-exchange chromatography could offer another approach for HES fractionation and, hopefully, once the functional activity of both weight and charge fractions will have been assessed, it will be possible to analyse a limited number of proteins by mass spectrometry.

For the glycan comparison, we first assessed the binding of seven biotinylated lectins available in the lab to the whole set of HES fractions (Fig. 23) to determine which lectin could be used to reveal glycans on 2D gels. Concanavalin A, which binds α -D mannose and α -D glucose residues, was the only one to show an increase in OD on fraction 14 compared to uncoated wells. Following this result, we ran two 2D SDS-PAGE of fraction 14 in parallel, silver stained one gel and performed a western blot for Con A on the other. Unfortunately, no specific Con A binding spot appeared, because of a very high non-specific binding background and this problem has not yet been solved.

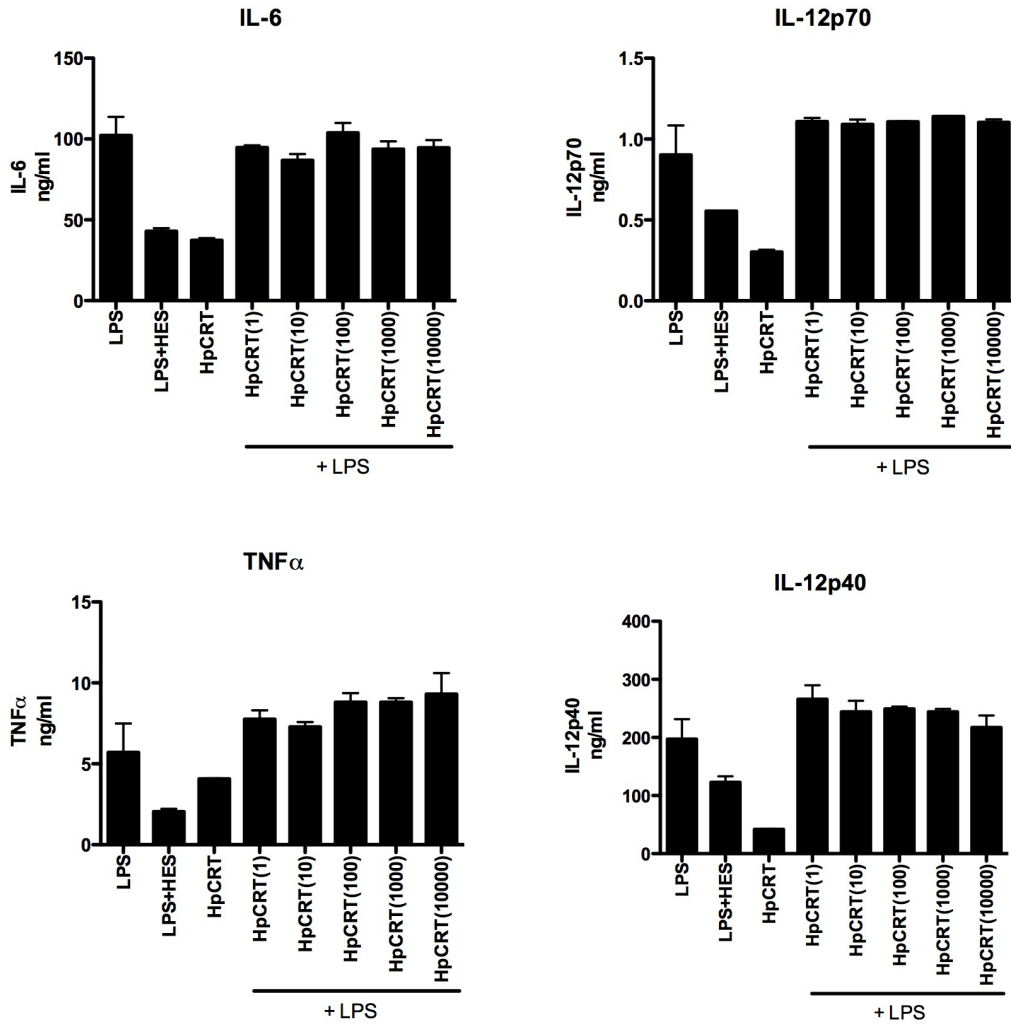


Figure 21 - Recombinant *H. polygyrus* calreticulin does not reduce LPS-induced inflammatory cytokines.

BMDCs were stimulated for 18 hours with 10 μ g/ml HpCRT or 1 μ g/ml LPS in presence or absence of 10 μ g/ml HES, 1, 10, 100, 1000, 10000 ng/ml HpCRT. Supernatant cytokines levels were measured by ELISA. Error bars represent the SEM of triplicate wells from the same BMDCs culture. This is representative of two similar experiments.

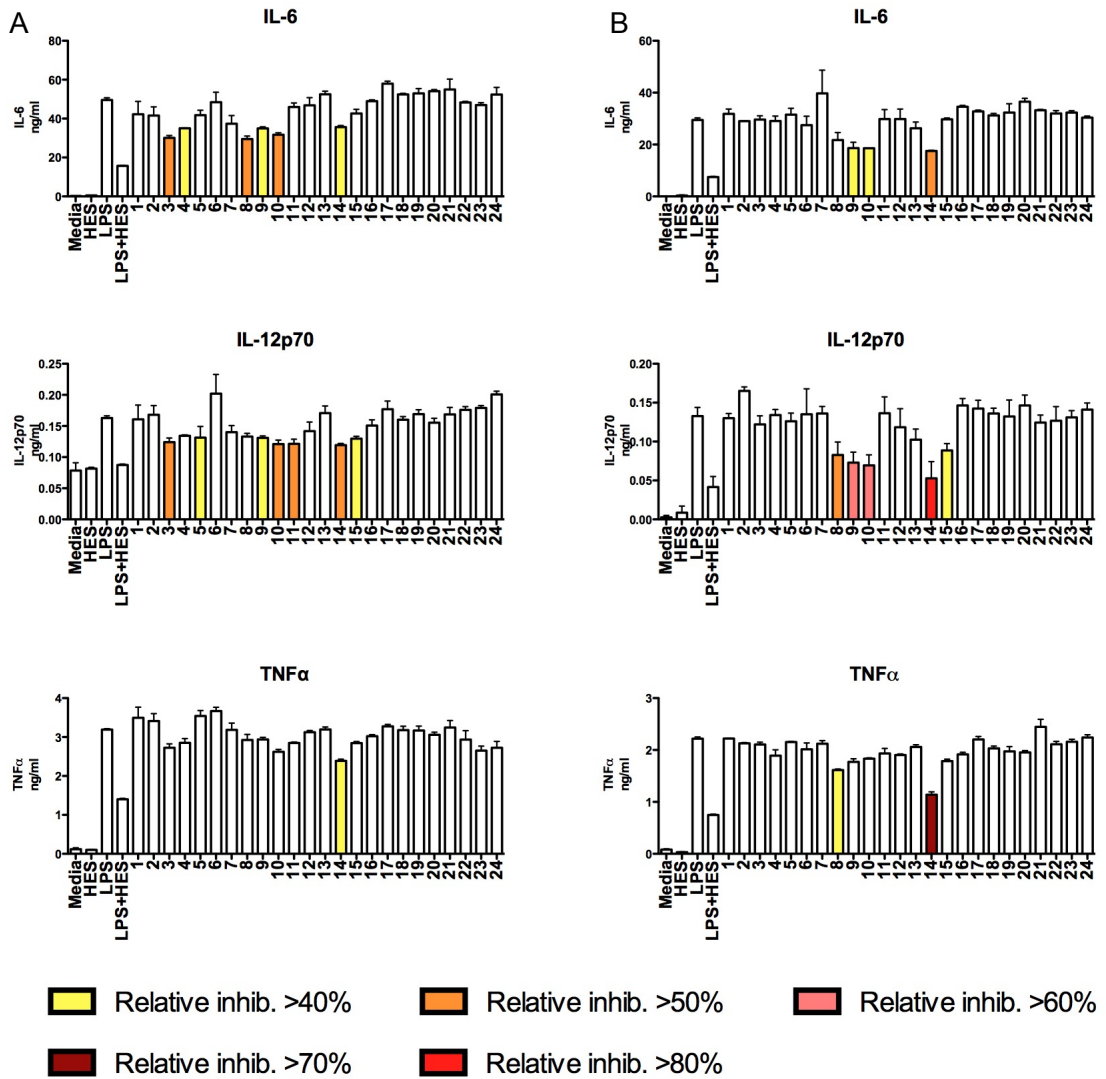


Figure 22 - HES size fraction number 14 is the most efficient in limiting the inflammatory cytokine production by LPS-stimulated BMDCs.

BMDCs were co-stimulated for 18 hours with 1 μ g/ml LPS and 10 μ g/ml HES or the approximate equivalent protein amount of each HES size fraction. Cytokines levels in the supernatants were measured by ELISA. Error bars represent the SEM of triplicate wells from the same BMDCs culture. Percentages of relative inhibition were calculated as the ratio of the difference between the cytokine level induced by the LPS and LPS/Fraction treated groups and the difference between the LPS and LPS/HES treated groups. Panels A and B display results from two independent experiments.

Fraction number	3	4	5	8	9	10	11	14	15
IL-6 BD-14	57	42	-	59	43	52	-	41	-
IL-6 BD-14	-	-	-	-	49	49	-	54	-
IL-12p70 BD-14	51	-	41	40	42	55	55	57	43
IL-12p70 BD-17	-	-	-	54	65	69	-	87	48
TNF α BD-14	-	-	-	-	-	-	-	44	-
TNF α BD-17	-	-	-	41	-	-	-	73	-

Table 3 - Summary of the percentages of total cytokine inhibition achieved by fractions with detected immunomodulatory activity.

Percentages rounded to the lower integer are displayed if higher than 40%. The formula used to obtain these numbers was:

$$\frac{\text{Cytokine level upon LPS treatment} - \text{Cytokine level upon LPS+HES fraction treatment}}{\text{Cytokine level upon LPS treatment} - \text{Cytokine level upon LPS+Total HES treatment}}$$

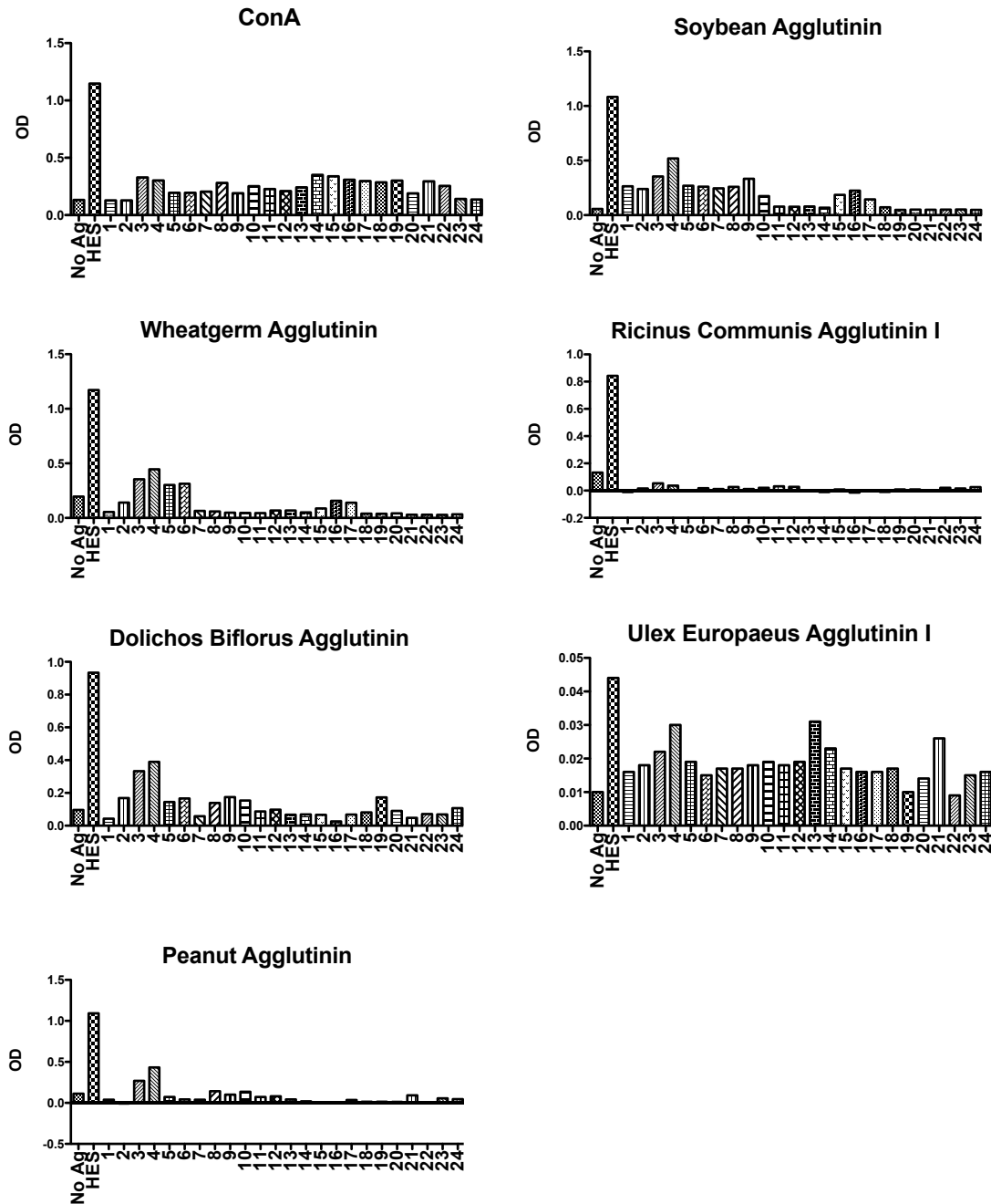


Figure 23 – Biotinylated lectins binding analyses on HES size fractions.

To detect highly expressed glycans in the different HES fractions, ELISA plates were coated with HES fractions, the estimated equivalent protein amount of HES or left uncoated. After a blocking step, biotinylated lectins were added and then revealed with the Strep-HRP/ABTS system. Optical density (OD) values of single wells for the fractions or mean of triplicate wells for the positive and negative controls were plotted.

3.6 HES binds CD24 on the surface of DCs, but the interaction with this molecule is not critical for the immune modulation.

The final method we used to study the molecular interaction between HES and DCs was to use biotinylated HES, provided by James Hewitson, for flow cytometry analysis on DCs. From the beginning the focus was CD24, a surface glycoprotein, which was shown by Hewitson to be the target of HES on B cells (unpublished data).

Preliminary analysis showed that biotinylated HES was also binding DCs and that this binding was correlated with CD24 positive cells (data not shown). Nevertheless, as nearly all our BMDCs expressed CD24, it was necessary to wait for CD24^{-/-} mice to confirm the binding of biotinylated HES to DCs through this molecule. As only two CD24^{-/-} mice were available before the end of the project, it was decided to go a bit beyond the scope of this work and also derive macrophages from this precious bone marrow. For simplicity, the same assay was performed on both cell types.

First, wild type and CD24^{-/-} bone marrow-derived macrophages (BMDMs) were stained for CD11b, CD11c, F4/80, MHC class II, CD40, CD80, CD86, CD24 and different concentrations of either biotinylated HES or biotinylated *H. polygyrus* extract (Hex), which is a simple homogenate of adult *H. polygyrus* worms. Biotin was then revealed with a second staining step with APC-conjugated streptavidin. When checking that macrophages were not activated and that the wild type and knockout populations were similar, it was noticed that the CD24^{-/-} BMDCs expressed slightly higher levels of F4/80 than the wild type, but all the other measured markers showed comparable levels of expression (data not shown). Then gating for live, CD11b⁺F4/80⁺ cells, the level of streptavidin binding was plotted (Fig. 24 and Appendix Fig. S 5 and S 6). A slight reduction of HES binding to CD24^{-/-} macrophages compared to wild type cells was seen, but the two titration curves remained very similar. Biotinylated Hex did not bind macrophages at all confirming that HES binding was specific. This result proves that HES interacts with BMDMs and suggest that the contribution of the interaction with CD24 is minimal in regard to the global binding. It should be mentioned here that James Hewitson performed the same analysis on B cells and showed that biotinylated HES was unable to bind CD24^{-/-} B cells, confirming that the interaction of HES with B cells was entirely CD24 dependent. Thus, it is certain that HES actually interacts with CD24, even though this binding activity does not seem very important on macrophages.

Because of this limited difference in the cell binding, no major divergence between the wild type and the knockout cells was expected in term of functional response to HES, but macrophages were nevertheless stimulated with LPS, LPS and a titration of HES or LPS and a titration of hiHES (Fig. 25). Similarly to the DC assay 10^5 cells were plated per well, but the assay was performed in flat bottom plates to allow for adhesion of macrophages. First of all, it must be pointed out that the levels of cytokines secreted by the macrophages in this setup were very low and no IL-12p70 was detectable. Moreover, CD24^{-/-} macrophages showed higher cytokines production upon LPS and HES co-treatment than upon LPS ligation only (Fig. 25). This should normally not occur and the experiment should therefore be repeated.

Nonetheless, one can still see a dose-dependent down-regulation of LPS-induced IL-6 by HES in both the wild type and the CD24^{-/-} group. The reduction of IL-6 level between the LPS group and the group treated with LPS and 10 μ g/ml HES was significant according to a Student's t test. Once again, hiHES did not show any dose-dependent effect. No significant variations of the other measured cytokines were observed even if there was a slight down-regulation of TNF α with 10 μ g/ml HES. After this assay, macrophages were stained for CD11b, F4/80, MHC class II, CD40, CD80 and CD24. The same kind of weak HES dose-dependent down-regulation of MHC class II and CD40 could be seen on both cell types (data not shown). This could be due to a technical failure in the purification process of the HES batch used in this experiment. However, if this trend is confirmed, it would suggest that the HES down-regulates inflammatory cytokine production of BMDM in a similar way it does for DCs and that this modulation is at least not fully CD24 dependent.

Second, wild type and CD24^{-/-} BMDCs were stained for CD11c, MHC class II, CD40, CD80, CD86, CD24 and different concentrations of biotinylated HES or biotinylated Hex. As for macrophages, DCs were found not to be activated and live, CD11c positive gated cell populations were similar. As expected, biotinylated HES bound wild type DCs in a dose-dependent manner, whereas the negative control, biotinylated Hex did not (Fig. 26 and Appendix Fig. S7 and S8). Interestingly, HES was able to bind CD24^{-/-} DCs, but the interaction was strongly reduced. Therefore, it appears that HES binds at least two molecules on DCs, one of them being CD24. This is really remarkable in regard to Liu et al. paper [53] and their hypothesis that interactions between DAMPs, CD24 and SiglecG could lead to an inhibition of the NF- κ B pathway.

Therefore, to assess if HES was down-regulating the production of inflammatory cytokines through CD24, we performed once again the usual immunomodulation assay on wild type and CD24^{-/-} BMDCs (Fig. 27). First of all, it was seen that, similarly to macrophages, CD24^{-/-} BMDCs were producing slightly less inflammatory cytokines than the wild type cells upon LPS stimulation, but none of these differences was significant according to Student's t tests. When co-stimulated, both groups displayed a HES, but not hiHES, dose-dependent inhibition of the inflammatory cytokines, suggesting that the immunomodulatory process is CD24 independent. However, for the same reason as in the macrophages assay, the immunomodulatory effects of HES were not as strong as expected from previous experiments. The inhibition of TNF α for example was not significant for both groups, nor was inhibition of IL-6 on CD24^{-/-} DCs. Thus, it will be needed to repeat these results in order to compare the efficiency of the HES inhibition on both DCs sets, since we cannot exclude that the HES immunomodulation could be weaker in the absence of CD24.

At the end of the incubation, DCs were stained for CD11c, MHC class II and co-stimulatory markers. Unfortunately, the LPS-pulsed wild type cells did not show a strongly activated phenotype and the HES induced immunomodulation was difficult to see. However, the assay worked well for CD24^{-/-} cells, which displayed a clear down-regulation of all the markers, suggesting that co-stimulatory markers could be modulated to the same extent in CD24^{-/-} and wild type DCs (Fig. 28). Once again, this must be confirmed in an experiment with a properly working wild type control.

In addition, the comparison of the biotinylated HES binding curves of macrophages and DCs (Fig. 25 and 27) indicated that the global avidity of HES compounds was higher for the macrophages than for the DCs. Moreover, the shift of the binding curve in the absence of CD24 seemed high for DCs, but minimal for macrophages. Together with Hewitson's results, these two experiments suggest that HES components bind CD24, but interactions with other receptors also occur. This or these receptor(s) are not expressed on spleen isolated CD90⁺ B cells, mildly expressed on GM-CSF grown BMDCs and highly expressed on M-CSF grown BMDM. In addition, CD24 ligation does not seem crucial for the ability of HES to modulate DC and macrophage activation states.

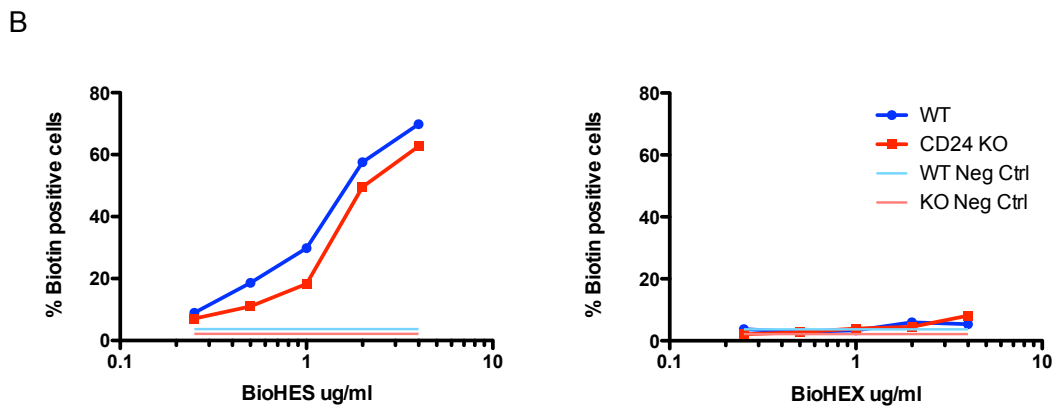
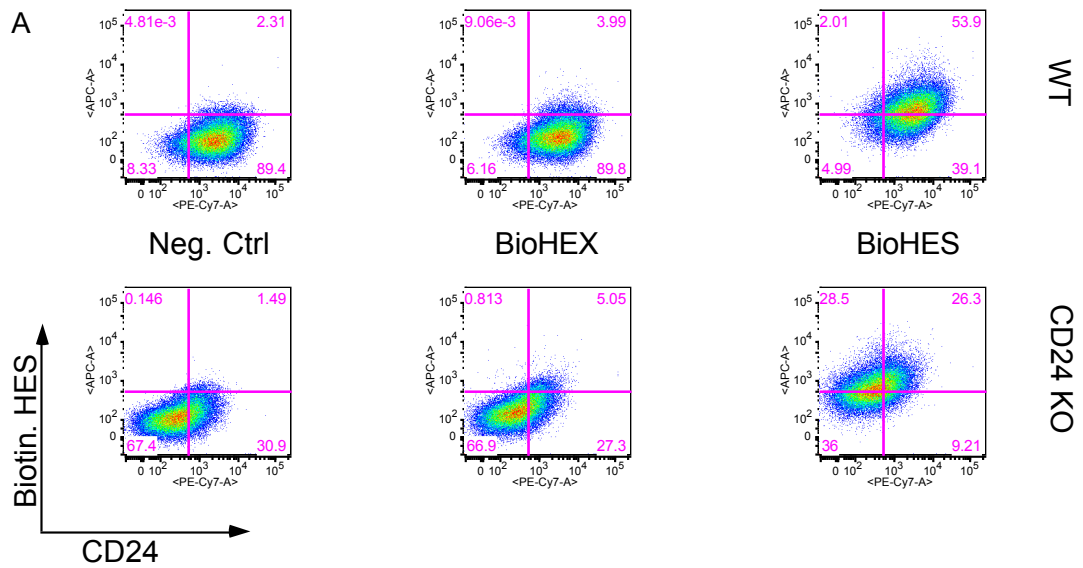


Figure 24 – Biotinylated HES binds to wild type and CD24^{-/-} bone marrow-derived macrophages in a similar manner.

Day 7 wild type and CD24^{-/-} BMDMs were stained for CD11b, CD11c, F4/80, MHC class II, CD40, CD80, CD86, CD24 and different concentrations of biotinylated HES or biotinylated Hex. The complete analysis is presented in appendix. Only live and CD11b⁺ F4/80⁺ gated cells treated with the top concentrations (4 μ g/ml) of biotinylated HES and Hex or no antigen are displayed here (panel A). HES or Hex binding on live CD11c⁺ cells, as determined by the percentage of biotin positive cells, was then plotted for the whole titration (panel B). Horizontal lines show the value of untreated controls.

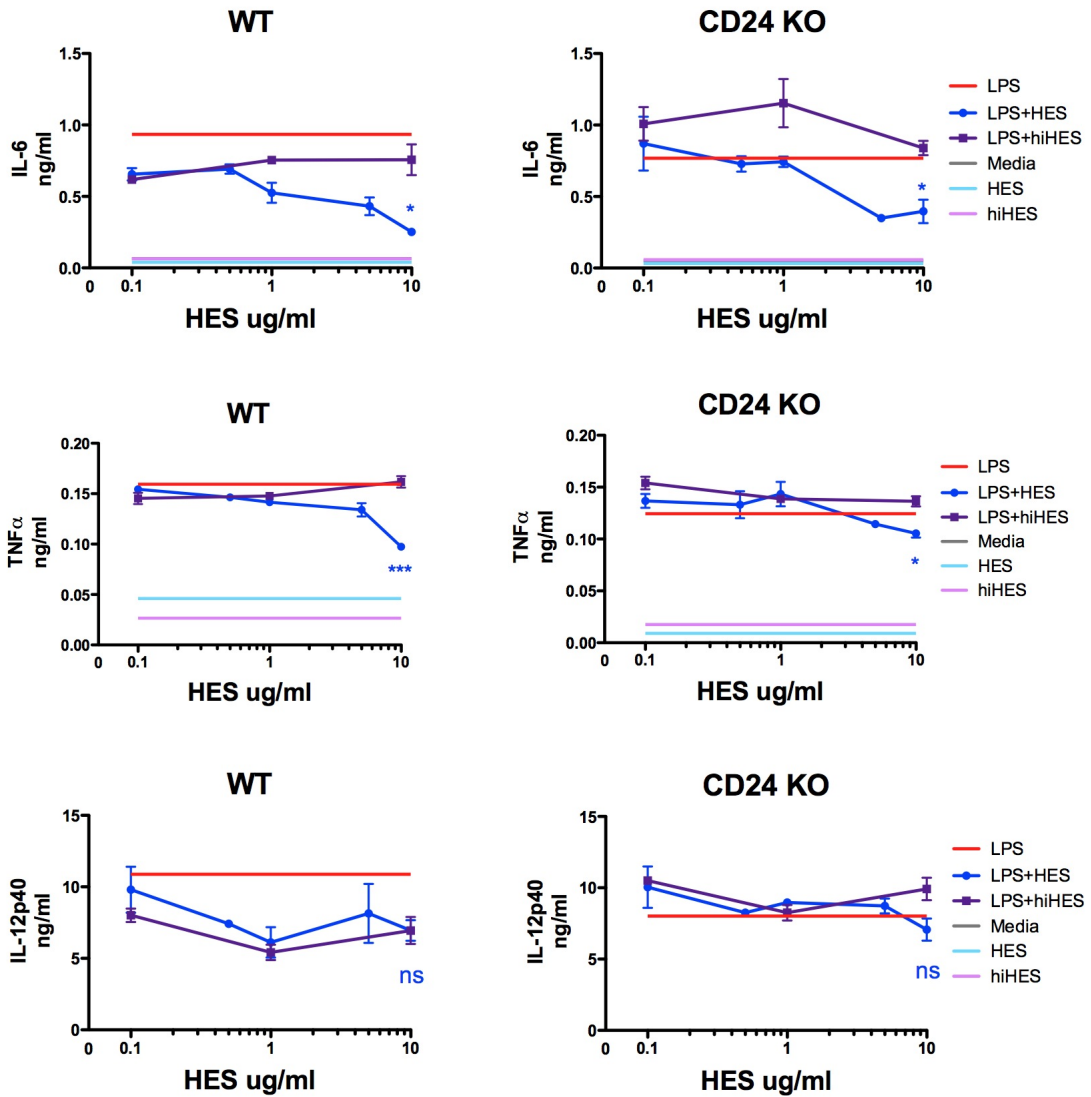


Figure 25 - Wild type and CD24^{-/-} bone marrow derived macrophages respond similarly to LPS and HES or hiHES co-stimulation.

Wild type and CD24^{-/-} BMDMs were stimulated with 1 μ g/ml LPS, 10 μ g/ml HES, 10 μ g/ml hiHES or a combination of LPS and various concentrations of HES or hiHES. 18 hours later, cytokines levels were measured in the supernatants by ELISA. IL-12p70 was not detected. Error bars represent the SEM of triplicate wells from the same BMDMs culture. Horizontal lines show the average of triplicate wells treated with one or no stimulus. Untreated controls did not produce a detectable level of TNF α . IL-12p40 was also below the detection threshold for the HES-, hiHES- and untreated controls. Statistics shown compare the LPS-treated group with the cells treated with LPS and 10 μ g/ml HES. Results of Student's t test: non significant (ns) $P > 0.05$, * $P < 0.05$, *** $P < 0.001$.

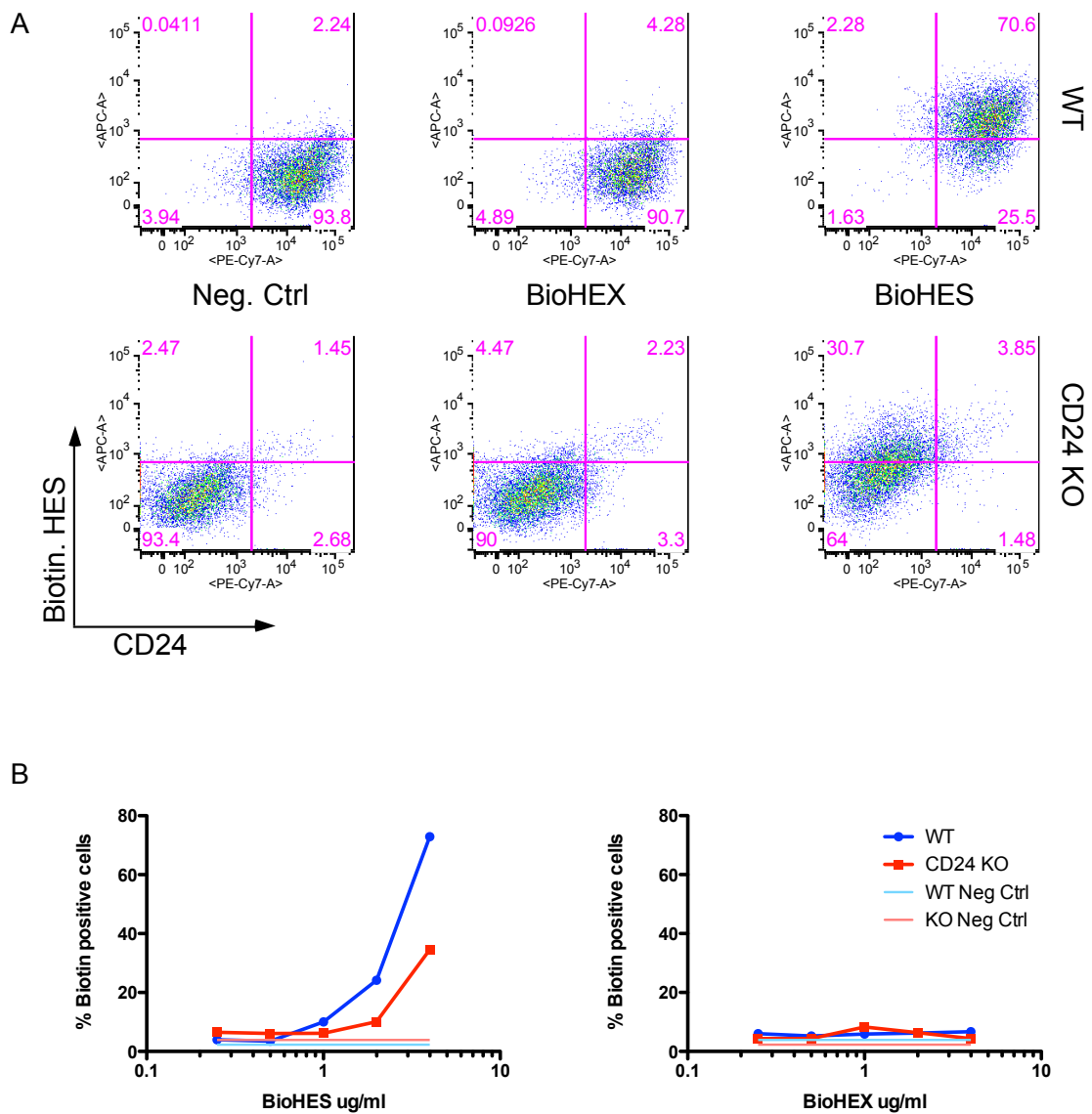


Figure 26 - CD24 deficiency strongly reduces biotinylated HES binding to BMDCs

Day 10 wild type and CD24^{-/-} BMDCs were stained for CD11c, MHC class II, CD40, CD80, CD86, CD24 and different concentrations of biotinylated HES or biotinylated Hex. The complete analysis is presented in appendix. Only live and CD11c⁺ gated cells treated with the top concentrations (4 μ g/ml) of biotinylated HES and Hex or no antigen are displayed here (panel A). HES or Hex binding on live CD11c⁺ cells, as determined by the percentage of biotin positive cells, was then plotted for the whole titration (panel B). Horizontal lines show the value of untreated controls.

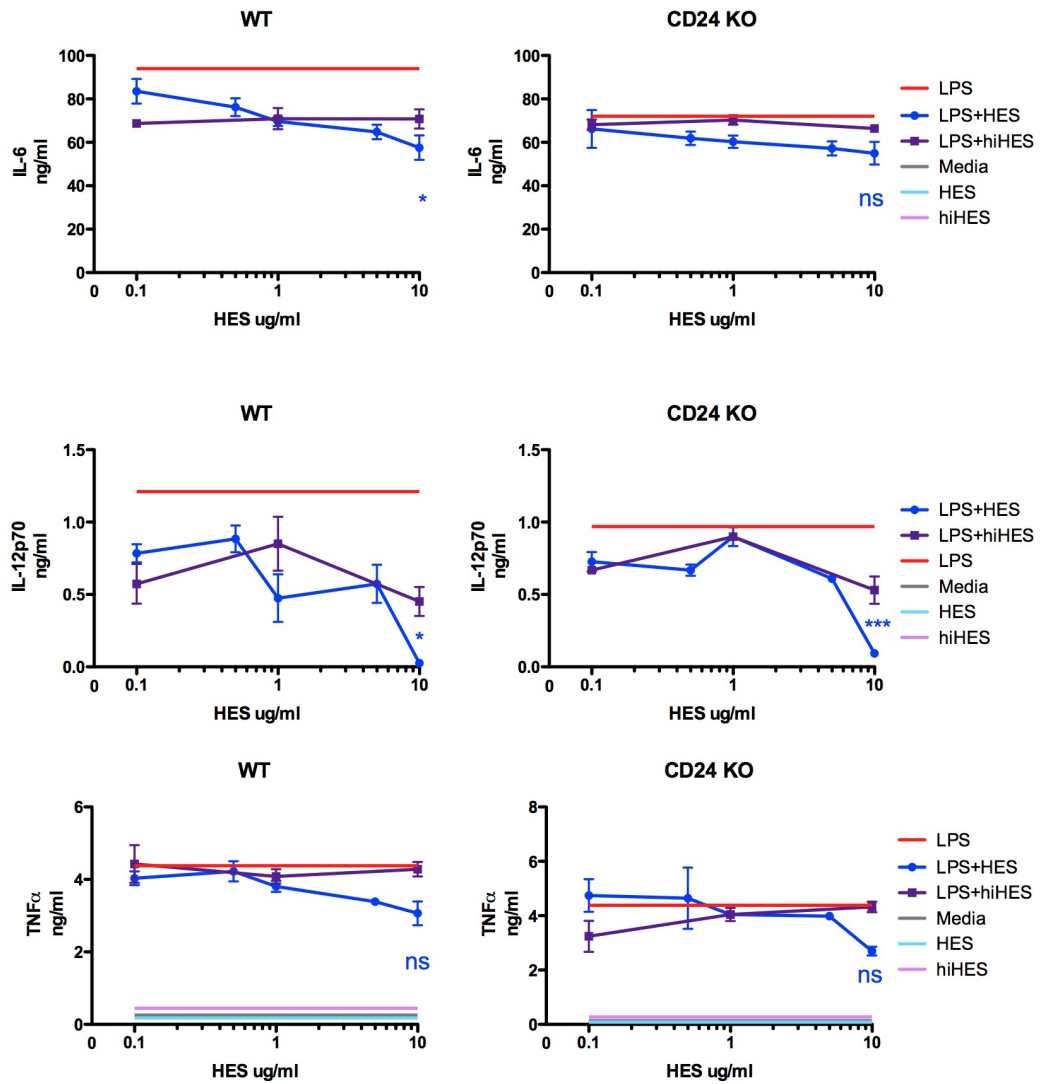


Figure 27 - HES, but not hiHES, down-regulates the inflammatory cytokines production of LPS-stimulated CD24^{-/-} BMDCs.

Wild type and CD24^{-/-} BMDCs were stimulated with 1μg/ml LPS, 10μg/ml HES, 10μg/ml hiHES or a combination of LPS and various concentrations of HES or hiHES. 18 hours later, supernatants cytokines levels were measured by ELISA. Error bars represent the SEM of triplicate wells from the same BMDCs culture. Horizontal lines show the average of triplicate wells treated with one or no stimulus. HES-, hiHES- and untreated cells did not produce detectable levels of IL-6 and IL-12p70. Statistics shown compare the LPS-treated group with the cells treated with LPS and 10μg/ml HES. Results of Student's t test: non significant (ns) P>0.05, *P<0.05, ***P<0.001.

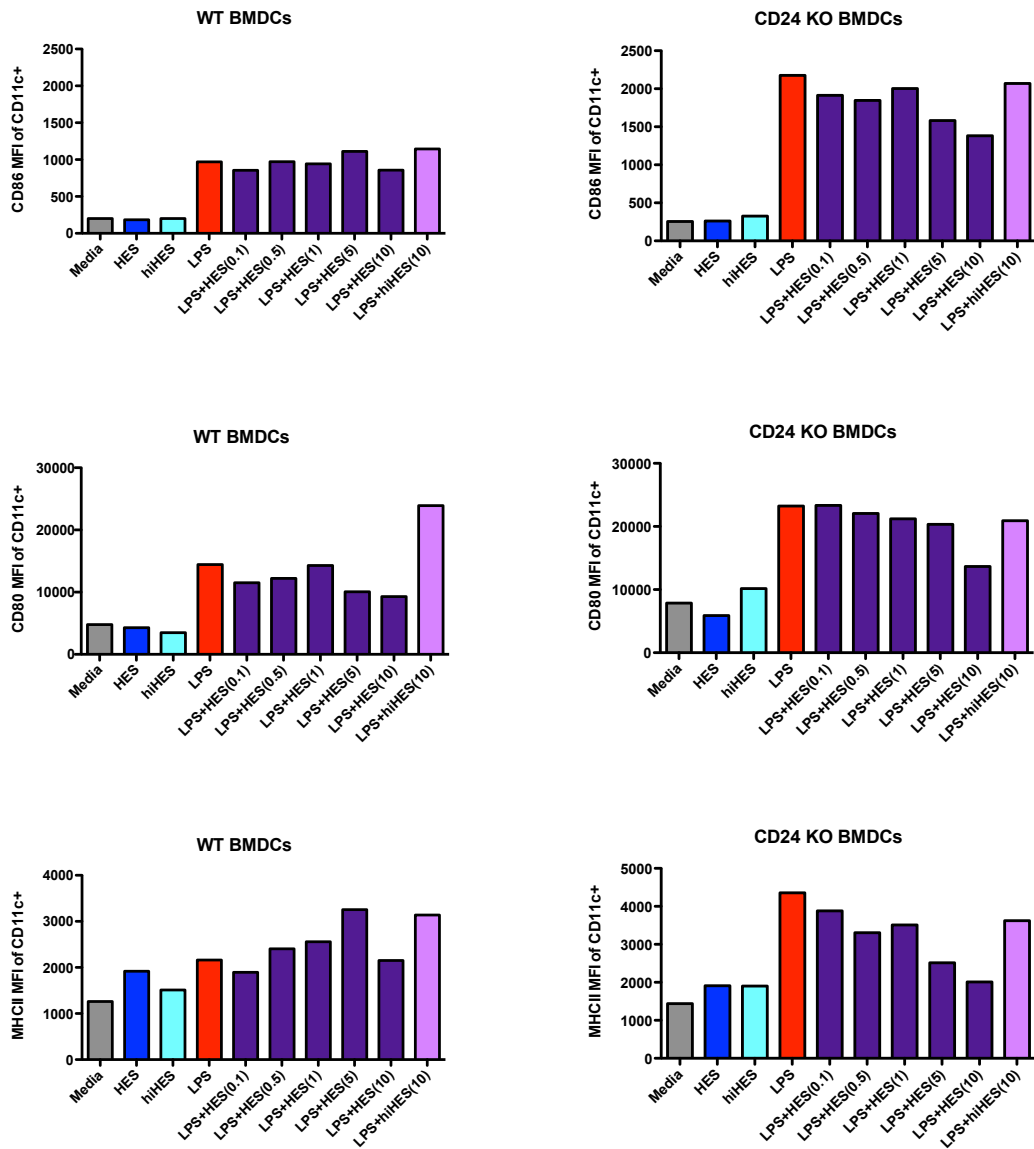


Figure 28 – HES inhibits LPS-induced up-regulation of co-stimulatory markers on the surface of CD24^{-/-} BMDCs.

After the assay described in figure 27, BMDCs were stained for CD11c, MHC class II, CD80, CD86. Gates were applied for live, CD11c expressing cells and the geometric mean of the fluorescence intensity for the other markers was plotted. Concentrations in brackets are in $\mu\text{g/ml}$. Single wells were stained and statistics are therefore not available.

3.7 DC adoptive transfer

Finally, we wanted to see if the in vitro modulations witnessed in this project were correlated with a downstream effector T cell response in vivo. In this view, we decided to ask three main questions. What kind of Th response profile does HES induce? Is HES able to modulate the Th response to a bacterial pathogen like it modifies DCs response to LPS and does heat-inactivated HES have the same properties as HES?

To answer these questions, we chose a model already used by John Grainger, based on the fact that DCs are able to differentially process several antigens by trafficking them in separate compartments [9]. First, we stimulated BMDCs with PBS, HES, hiHES, heat-inactivated *P. acnes* extract (Pa) or a combination of these antigens for 18 hours. Killed *P. acnes* extract was the typical Th1 inducer used in Cervi et al.'s paper [9] to stay closer to the infection setup, where DCs are exposed to whole bacteria rather than only one of their many cell wall components. Then, we checked that Pa, which contains multiple different PAMPs, induced DCs activation in the same way as LPS did in the in vitro assays. Co-stimulatory marker expression and cytokine production were measured at the end of the incubation and the activation of DCs by Pa, the tolerance of HES and hiHES as well as the ability of HES, but not hiHES, to inhibit Pa-induced DCs activation were confirmed (Fig. 29). Next, we washed the harvested DCs and adoptively transferred them into the peritoneal cavity of naïve BALB/c recipients. One week later, T cells were recovered from spleens and draining lymph nodes and re-stimulated with an anti-CD3 antibody to measure the polyclonal response, or with HES or Pa to assess the antigen specific response. After 72 hours incubation, cytokine levels were measured in the supernatants.

The variability of the outcome of the in vivo transfers was high and it is difficult to argue that the results presented here show any real immunomodulation because Pa never really induced the expected strong Th1 response. Nonetheless, we will try to identify the dominant patterns that were conserved throughout the experiments.

First, in the lymph nodes, no significant antigen-specific response was detected, suggesting that either the numbers of DCs injected were not sufficient or that these cells were unable to properly migrate to reach and activate T cells. Accordingly, the polyclonal response was not strongly polarised even though the HES- and hiHES-pulsed group tended to be dominated by Th2-related cytokines, whereas Pa pulsing clearly induced a Th1/Th17 type response (Fig. 30). To simplify, IL-5, IL-13 and IL-

10 graphs, which displayed similar patterns to IL-4, were not shown on this figure. Both co-pulsed groups showed intermediate responses, with relatively high levels of all measured cytokines except for IFN γ that was down-regulated compared to the Pa-pulsed group, suggesting that both HES and hiHES are able to limit the development of a Th1-type response. However, this down-regulation was not significant.

In the spleen, the non-specific response to HES- and hiHES-pulsed DCs seemed to induce Th2-related cytokine production, but this was not significant compared to the α CD3-induced response of the un-pulsed DCs group, since this group produced relatively high levels of all the measured cytokines without particular polarisation (Fig. 31). Nevertheless, the levels of IL-4 produced by HES- and hiHES-pulsed DCs-activated T cells were significantly higher than the IL-4 production of the Pa-pulsed group, whereas their IL-17 and IFN γ production was low. Together, these results suggest that both HES- and hiHES-pulsed DCs are able to polarise T cells so that they tend to induce a Th2-type response once they are activated by a non-specific stimulus. In this setup, it could also be interesting to compare the response of non-injected mice to the response of mice injected with un-pulsed DCs to see if the DCs injection by itself is the cause of the relatively high cytokine levels in response to anti-CD3.

T cells activated by Pa-pulsed DCs did not produce Th2-related cytokines, but induced a strong Th17 response. However, and as opposed to what we saw in the lymph nodes, they did not stimulate the expected Th1 response. The Pa-pulsed group was strongly heterogeneous with two mice that did not respond at all and two mice that were producing very high levels of IFN γ even in the absence of re-stimulating antigen. If we look at the only other experiment where anti-CD3 re-stimulation was performed (Fig. 32), it seems that Pa-pulsed DCs polarise T cells toward a Th1-type response. This let us hope that, after an optimisation of the protocol, a strong Th1 induction by Pa could be achieved in this model and therefore allow for a convincing assessment of the HES and hiHES ability to skew the Th1/Th2 balance upon adoptive transfer of co-stimulated DCs. The same figure also suggests that HES is able to inhibit the Th1 response to *P. acnes*. Unfortunately, the hiHES and hiHES/Pa groups were lacking in this experiment because of the poor yield of that particular BMDCs culture, but, on figure 31, we see that the hiHES/Pa co-pulsed group shows the same response as the HES/Pa group, suggesting that they could behave in a similar way.

The two co-pulsed groups showed a mixed Th response. Their levels of IL-4 were reduced compared to the HES- and hiHES-pulsed groups, but higher than the Pa-pulsed group. No induction of IFN γ was detectable, but neither HES nor hiHES seemed able to decrease the Pa-induced Th17 response.

IL-17 was the only measured cytokine that showed strong responses upon antigen-specific re-stimulation (detailed on Fig. 31B). Similarly to the polyclonal response, Pa re-stimulation induced similar levels of IL-17 in the Pa-pulsed and in both co-pulsed groups, confirming the inability of HES or hiHES to modulate the Th17 response to Pa. Interestingly, HES re-stimulation even caused an increase of IL-17 levels in the HES and HES/Pa-pulsed, but not the hiHES and hiHES/Pa-pulsed groups. The unexpected HES-specific IL-17 production in the HES-pulsed group was not repeated in any other experiments. On the contrary, the same effect in the HES/Pa co-pulsed group was to see in three out of the four transfer experiments. The fact that the HES-specific IL-17 production was higher than the Pa-induced level in the HES/Pa co-pulsed group, but not in the hiHES/Pa group suggest that the heat-labile components of HES could be able to skew the immune response toward a Th17 rather than a Th1 response in the physiologically relevant case of the simultaneous uptake of both *H. polygyrus* and bacterial products by DCs.

The regulatory response-associated IL-10 production was similar to the IL-4 pattern (data not shown). Anti-CD3 induced high levels of IL-10 in both HES- and hiHES-pulsed groups, low levels in the Pa group and intermediate levels in the co-pulsed groups. Together, these results suggest that HES-pulsed DCs and *P. acnes*-pulsed DCs polarise T cells toward different Th profiles and that HES as well as hiHES can modify the response to *P. acnes* upon co-treatment. Moreover HES, but not hiHES, seems able to favour a Th17 rather than a Th1 response upon DCs co-stimulation.

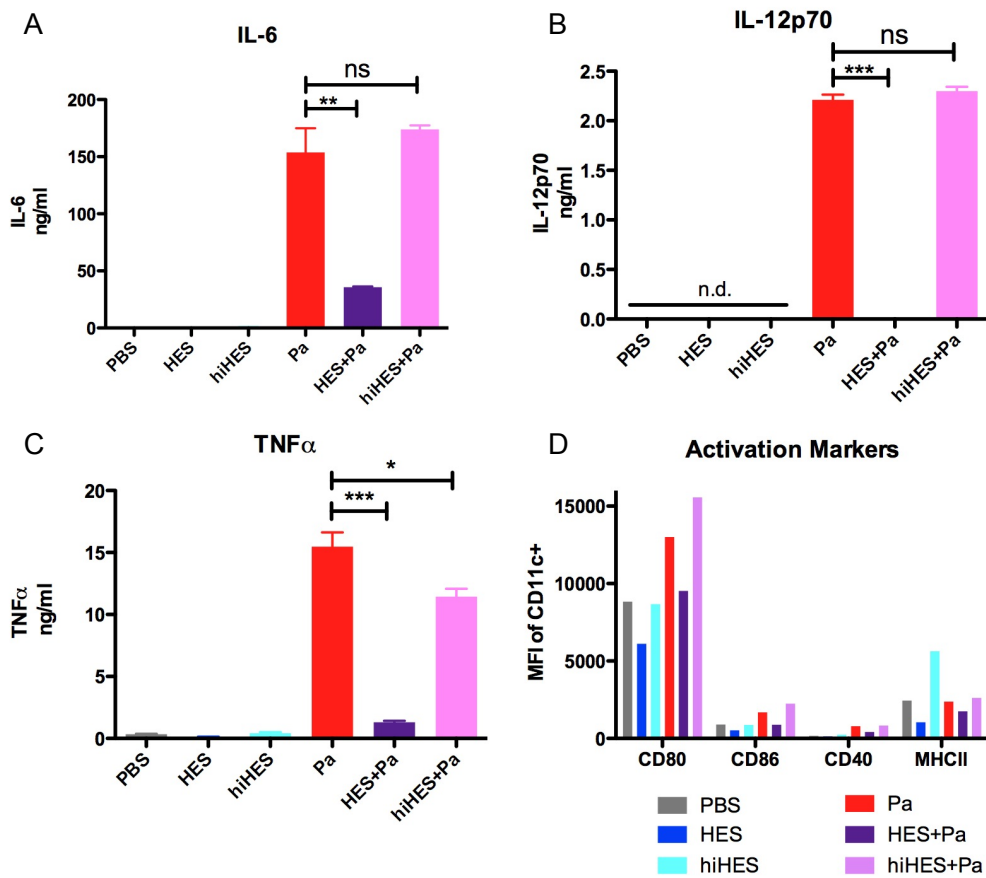


Figure 29 – *P. acnes* activates DCs similarly to LPS and HES, but not hiHES, is able to inhibit this activation.

With the aim of transferring them in vivo, BMDCs were stimulated with 10 μ g/ml *P. acnes* extract, 25 μ g/ml HES, 25 μ g/ml hiHES or a combination of Pa and HES or Pa and hiHES. After 18 hours, supernatant were harvested and cytokines concentrations were measured by ELISA (panel A, B and C). Error bars represent the SEM of triplicate wells from the same BMDCs culture. n.d. stands for not detected.

DCs were washed and injected into BALB/c recipients and the left over cells were stained in single wells for their surface expression of CD11c, MHC class II, CD80, CD86 and CD40. A gate for live cells expressing CD11c was applied and the geometric mean of the fluorescence intensity of the other markers was plotted (panel D). Results of Student's t test: non significant (ns) $P > 0.05$, $**P < 0.01$, $***P < 0.001$. Data are representative of four similar experiments.

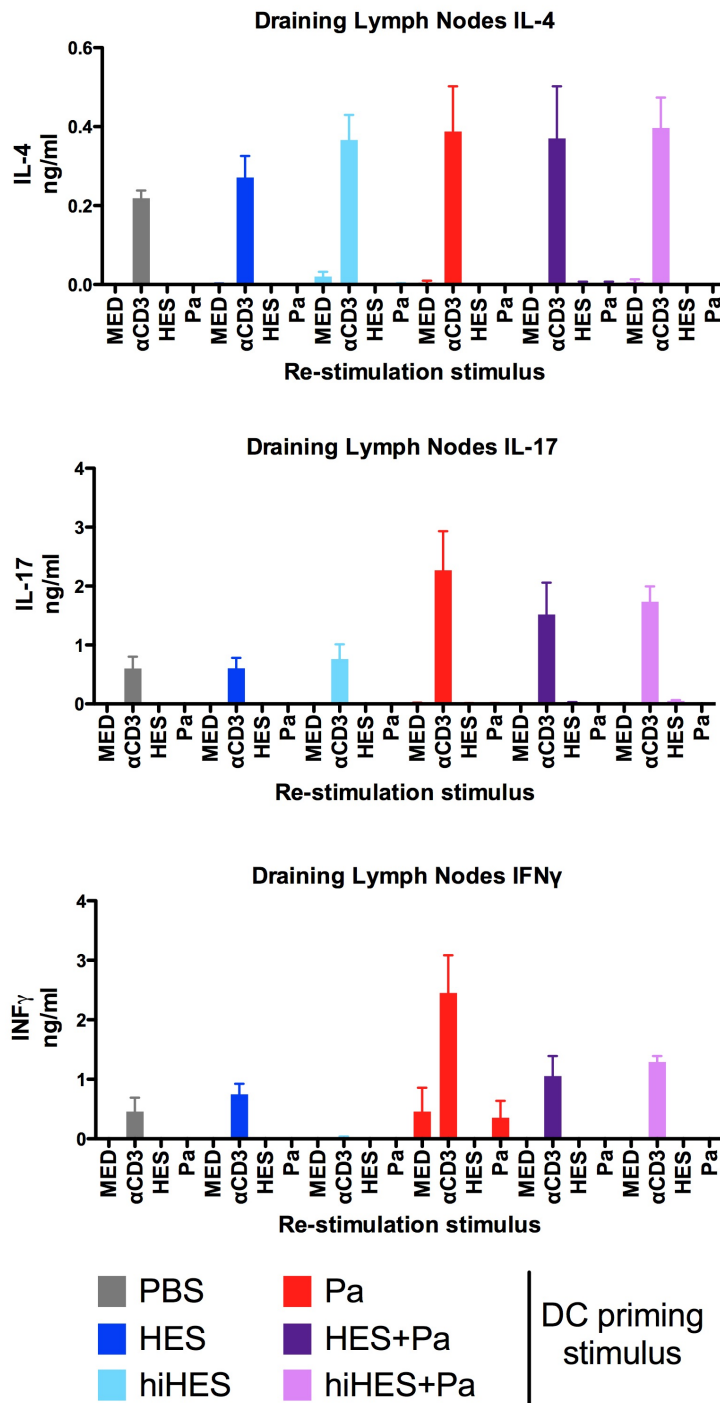


Figure 30 - Polyclonal and antigen-specific Th responses induced in the draining lymph nodes by adoptive transfer of HES-, hiHES-, Pa- and co-pulsed DCs.

BMDCs stimulated following the protocol described in figure 29 were injected intraperitoneally in naïve BALB/c recipients. Seven days later, draining lymph nodes of the peritoneal cavity were mashed and the cells were re-stimulated with 2 μ g/ml anti-CD3 antibody (α CD3), 10 μ g/ml HES, 1 μ g/ml Pa or left in media. After 72 hours, cytokine levels in the supernatants were measured by ELISA. Error bars represent the standard deviation (SD) of four different animals. IL-5, IL-13 and IL-10 production (not shown here) was very similar to the IL-4 pattern.

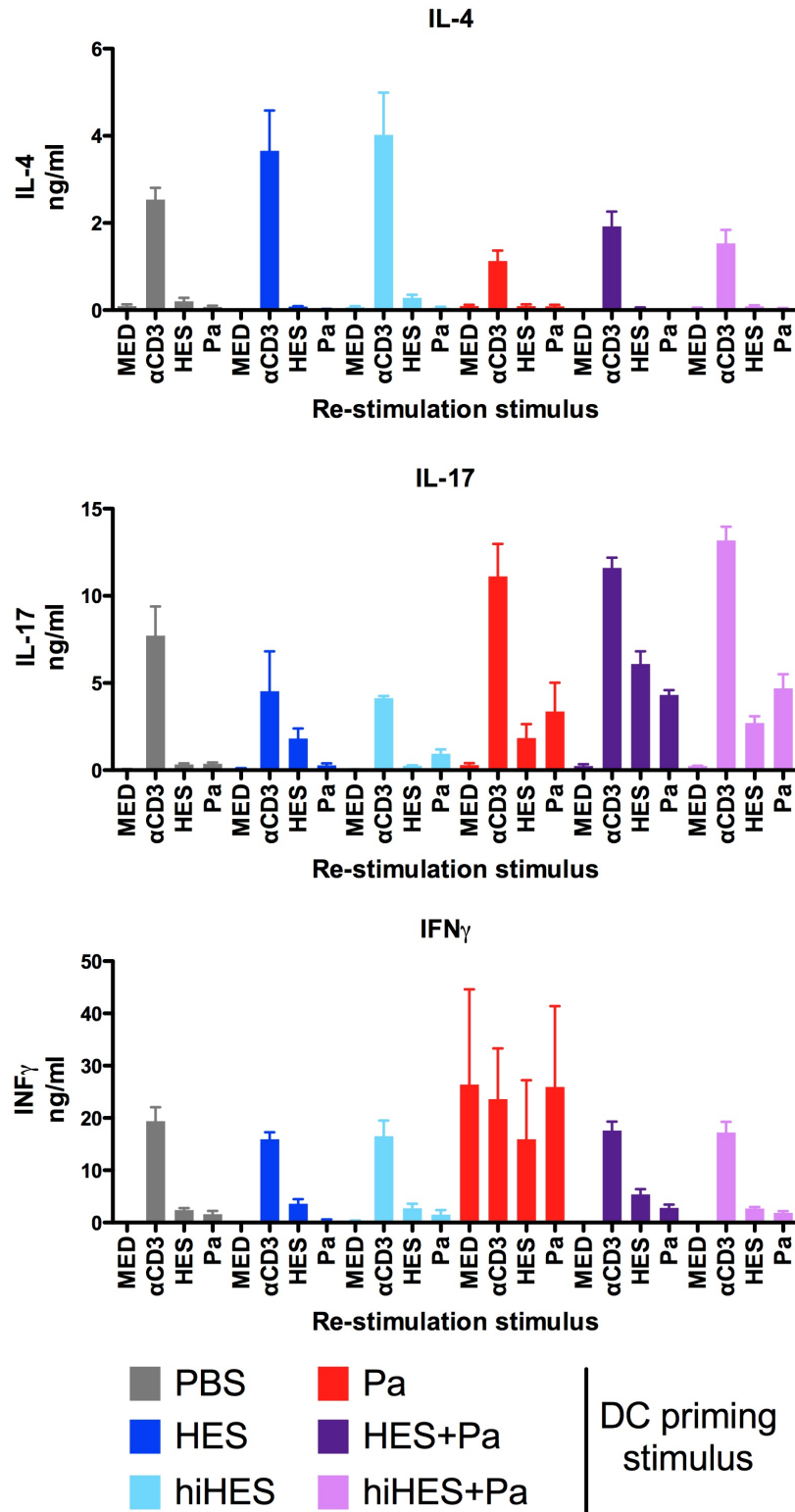


Figure 31A - Polyclonal and antigen-specific Th responses induced in the spleen by adoptive transfer of HES-, hiHES-, Pa- and co-pulsed DCs.

Following the protocol described in figures 29 and 30, splenocytes were re-stimulated for 3 days and cytokine levels in the supernatants measured by ELISA. Error bars represent the SD of four different animals. IL-5, IL-13 and IL-10 production (not shown here) was very similar to the IL-4 pattern.

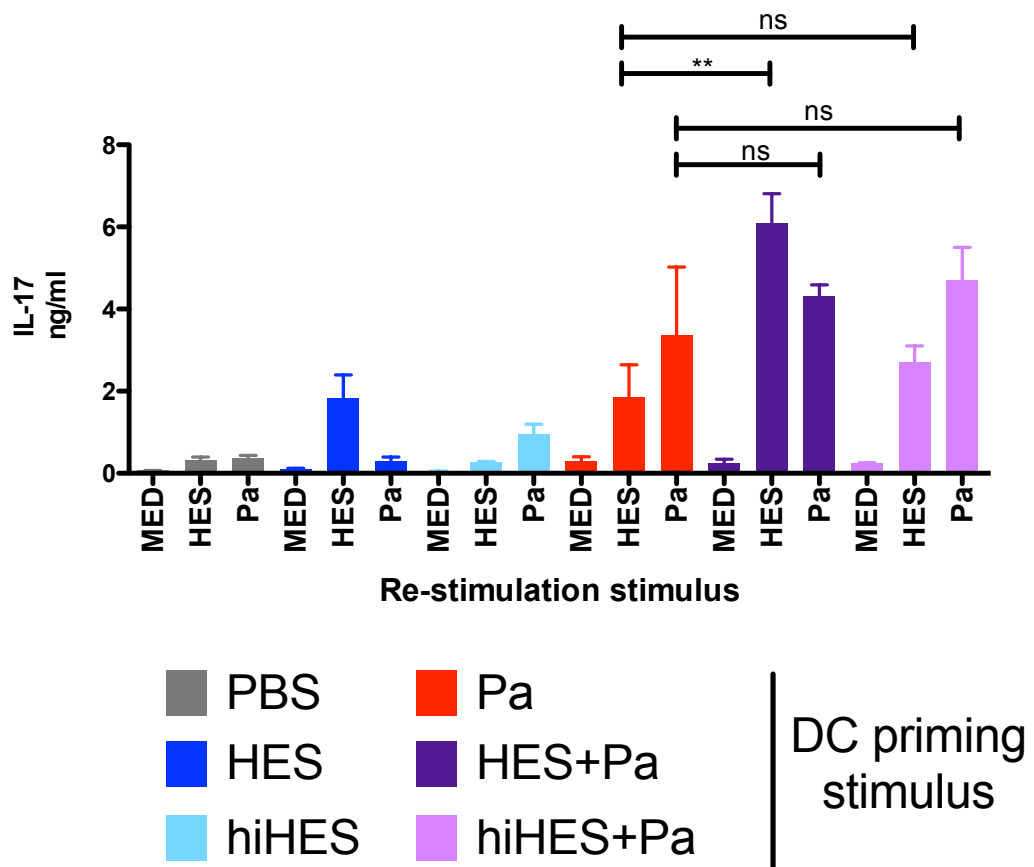


Figure 31B - Detail from figure 31A showing the IL-17 production in response to antigen-specific re-stimulation.

Results of Student's t test: ** P<0.01, non significant (ns) P>0.05

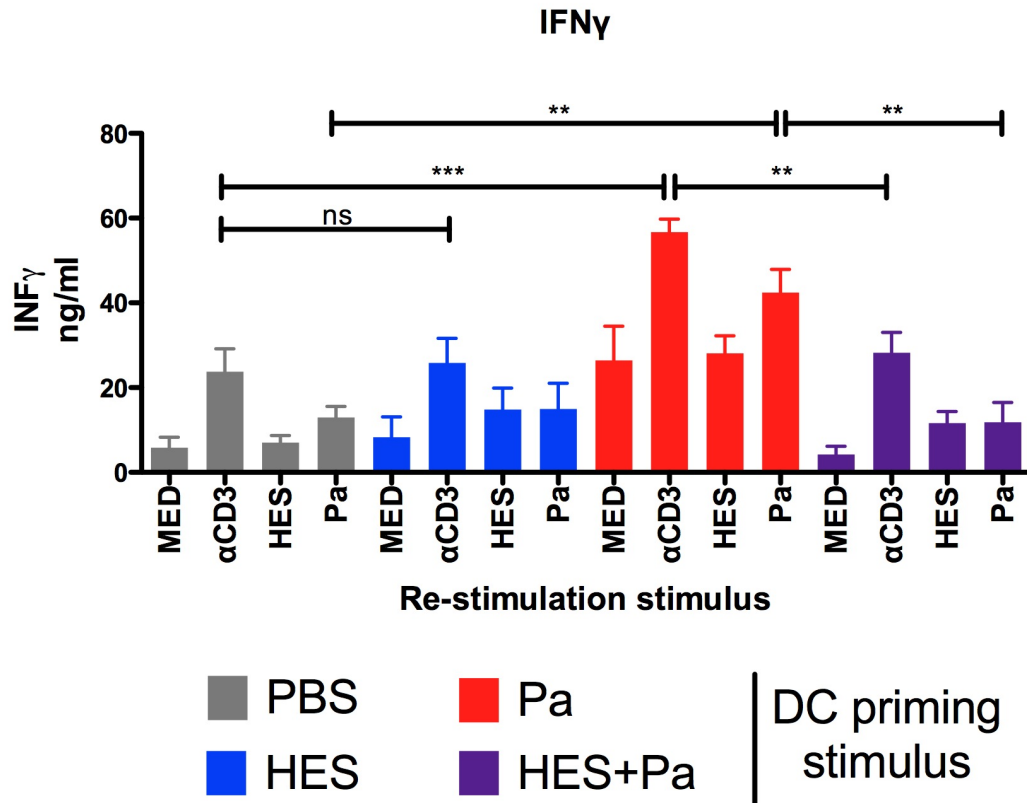


Figure 32 - Splenocyte interferon γ response to HES-, Pa- and co-pulsed DCs from a separate experiment.

The exact same protocol as in figure 31 was followed in this experiment lacking the hiHES and hiHES/Pa groups, but showing the expected Th1 response induced by Pa-pulsed DCs. Error bars represent the SD of four different animals. The first part of the label gives the DC priming stimulus. The second part shows the re-stimulation stimulus. Results of Student's t test: ** $P < 0.01$, *** $P < 0.001$, non-significant (ns) $P > 0.05$.

4 Discussion

The study of the interactions between a parasite and its host are as fascinating as the mechanisms behind them are complex. The strategies applied by both organisms are the result of a very long co-evolution during which one species had to adapt to any new feature of the other. Therefore, the molecular interactions can be quite intricate and difficult to understand, but most of the times the final result tend to an equilibrium that allows both organisms to survive.

In the case of intestinal helminth infections, the goal of the worms is to keep the immune system in a low state of activation in order to avoid being expelled from the gut. Inducing a regulatory response seems a good way to go and *H. polygyrus* has indeed developed this ability [39]. Nevertheless, some level of effector response will always remain, as foreign antigens will be presented to T cells. The definition of the best T helper environment from the worm's point of view is a bit more problematic. A Th1/Th17-type response is definitely one to avoid, as it would induce tissue damage potentially allowing bacterial infection and would be equally harmful for the host and the parasite that would then have to deal with a highly inflammatory environment. On the other hand, a Th2-type response would maintain the integrity of the gut, but is likely to lead to the worm expulsion through increased mucus secretion and peristaltic movements of the intestinal smooth muscles [54]. Therefore, the survival of a worm in a particular animal for an extended period suggest that this parasite has managed to skew the immune response of its host, in order to maintain the perfect balance between tolerance, inflammation and expulsion.

From the host point of view, mounting an efficient Th2 response seems crucial to expel the worms, at least in the *H. polygyrus* model [4, 55, 56]. However, as gut parasites are not proliferating inside their host, they do not represent an immediate life-threatening condition. Therefore, maintaining low levels of inflammatory cytokines at the invasion site is actually also beneficial to the host, as this will avoid a chronic pathogenesis linked with extended tissue damage. Thus, host and parasite have a common interest in keeping the Th1 response low, but have different aims in regard to the Th2 response. This could explain why the excretory/secretory products of *H. polygyrus* are so efficient in down-regulating the inflammatory cytokine production of DCs as both organisms actually benefit from a local anti-inflammatory environment in the gut mucosa. This down-regulation could thus as well be seen as an optimised host response to the parasite.

As DCs have been shown to be crucial in the induction of a Th2-type response [57], the question is now to know if HES can modify their ability to induce Th2 cells. In the acute intestinal infection model *Nippostrongylus brasiliensis*, in vivo transfer of excretory/secretory-pulsed BMDCs have been reported to induce a strong specific Th2 response [58], which may be the cause of the quick elimination of the worms. Although *H. polygyrus* is able to invade mice for an extended period, John Grainger described a similar Th2 induction upon HES or hiHES-pulsed BMDCs transfer and HES re-stimulation. In order to have an in vivo assay available to test potential HES immunoactive molecules or DCs deficient for some key molecules, we repeated Grainger's work, but when we transferred HES-pulsed DCs intra-peritoneally, we were not able to detect a strong antigen-specific Th2 cytokines production upon spleen or draining lymph node T cell re-stimulation. This could be due to several parameters that should still be optimised, like the number of DCs injected that was sufficient for Grainger's experiments in C57BL/6, but may not be in BALB/c. Another issue could be the ability of DCs to reach the lymph nodes. Thus, it would be a good idea to repeat that experiment with fewer groups and titrate the number of DCs injected. In addition, injections in the footpad followed by re-stimulation of popliteal lymph nodes could be tried. We already used this model with another protocol of DCs pulsing that did not turn out to work, but this type of injection at least showed a huge increase of the size of popliteal lymph nodes suggesting a good migration of the DCs. It would be interesting as well to pulse DCs with another strong Th2 inducer like *Nippostrongylus brasiliensis* excretory/secretory products (NES) or SEA to compare their ability to induce Th2-related cytokines in this setting with the HES-induced Th2 cytokines.

Concerning the non-specific response induced by anti-CD3 re-stimulation, the general pattern seems to fit the hypothesis stating that HES induces a Th2-type response with some regulatory features. The Th1 induction expected with Pa-pulsing was not achieved, and, for this reason, we cannot argue that the HES co-treatment reduces the Pa induced Th1 response in the spleen. However, it repeatedly did in the lymph nodes as well as in the spleen in a previous experiment (Fig. 32). On the other hand, HES was clearly unable to modulate the Pa-induced IL-17 production.

Thus, even if not perfect, taken together, these results seem to indicate that HES and hiHES possess immunomodulatory properties in our in vivo transfer model. HES and hiHES-pulsed DCs were able to polarise T cells toward a Th2-type response. However, this response was only seen upon non-specific re-stimulation

and the HES-specific response gave very different outcomes throughout the experiments, indicating that the experimental setup still needs improvements. The ability of HES to skew the response to a bystander antigen toward a less inflammatory one was also suggested even though not proven. Interestingly, the immunomodulatory properties of hiHES, that induced the same in vivo effects as HES with the exception of HES-induced IL-17, but did not affect the post-incubation activation state of DCs, proves that the cytokine production profile and co-stimulatory markers expression are not directly correlated with the final outcome of the adaptive immune response. This suggests that the DC response to *H. polygyrus* products is dynamic, with outcomes at the infection site during the first hours of contact with the pathogen products different from the later effects in the lymph nodes when T cells are activated. Whether this is an optimised response of the mouse or a real immunomodulation by the pathogen remains to be determined.

Even if the real impact of HES on the adaptive immune response to *H. polygyrus* needs further studies to be fully understood, we would like to focus now on the molecular aspects of the interactions between HES and dendritic cells, as this was the main topic of this project.

In this work, we confirmed that the incubation of bone-marrow derived DCs with HES does not affect the activation state of the cells as measured by their cytokine production and co-stimulatory surface marker expression, but that the addition of this parasite product on DCs activated with different TLR ligands limits the expression of those markers as well as the production of TNF α , IL-6 and IL-12, especially the active heterodimeric form referred to as p70. This strong reduction of cytokine levels is an ideal read-out for functional assays on HES in the search for the immunoactive product(s) because it allows one to clearly differentiate total, partial or null effects. Moreover, DCs are crucial in the early and late innate response to parasites, since their cytokine production profile will locally affect the lamina propria environment by modifying the inflammation state of the tissue and attracting or not other immune cell type. DCs also migrate to lymph nodes where they will interact with T cells and therefore polarise the adaptive response. In addition, the stimulation of DCs with products such as LPS or CpG is relevant to the real infection by intestinal helminths, since these parasites alter the gut structure and permeability [59] and therefore expose DCs to bacterial products. So, this model has great advantages and should be used for further studies, but unfortunately, it also has drawbacks. First, the in vitro culture provides only one type of DCs which is perfect for in vitro assays, but do not represent the complexity of the

dendritic cells populations present in the intestinal micro-environment. For example, we have, for now, no idea of the response of plasmacytoid DCs to *H. polygyrus* products. Second, the opportunity to transfer DCs in vivo may be more relevant to the actual *H. polygyrus* infection than in vitro assays, but it could be biased toward a Th2- type response induction when naïve DCs are injected. Thus, a reliable negative control is lacking. Finally, a major problem during this project was the achievement of a reproducible phenotype and a decent yield from the in vitro cultures. Apparently, even very minor changes in the protocol had an impact on the cell phenotype and one more substantial change even affected the cytokine production. Therefore, in order to obtain comparable results, the greatest care must be given to the BMDC culture.

Once we had a well-characterised model, we considered two approaches to our problem. As for any host-parasite interactions study, we could either focus on the host, in our case the dendritic cells, or on the parasite, here more specifically its excretory/secretory products. First, we investigated three signalling pathways based on evidence of their implication in immunomodulatory processes in other parasite models or in other cell types. The TGF β pathway was the most obvious target as Grainger reported its fundamental role in the *de novo* induction of Foxp3 in CD4⁺ T cells [39]. However, our results strongly suggested that this pathway is not used to modulate DCs cytokines production. Of course, as mentioned before, further work on TGF β receptor deficient DCs is needed to definitely prove this conclusion. In addition, this does not mean that the TGF β homologue present in HES does not affect DCs at all. It could for example, affect pathways that do not alter the activation state of the DC directly, but could modify antigen presentation or other interactions with any kind of immune cells, resulting in a modified systemic response like the alteration of the response to *P. acnes* we described before. Again, a knockout model would be ideal to study this possibility.

Then, we looked at the implication of the C-type lectin receptors. Since we had not enough time to study them all, we chose to investigate the role of Dectin-1 and -2 and the ITAM-binding kinase Syk that offers the opportunity to target several CLR, including Mincle, CLEC-2 and BDCA-2 [60, 61], in one experiment. The choice of Dectin-2 was motivated by a paper by Ritter et al. [31] showing interactions between this CLR and *Schistosoma mansoni* antigens whereas Dectin-1 was reported to modulate the cytokine response to fungal products [45]. No recovery of cytokine levels was seen upon antibody-mediated blockage of both Dectin-1 and Dectin-2 or in the Dectin-1^{-/-} DCs. Moreover, the two Syk inhibitors tested failed to enhance the

cytokine production upon LPS and HES co-treatment. Therefore, on top of ruling out roles for Dectin-1 or -2 in the DCs modulation by *H. polygyrus* products, we can also suggest that Syk signalling, and thus other CLR s like Mincl e, CLEC-2 or BDCA-2, are not needed.

Nevertheless, the C-type lectin receptor family remains a major target for immunomodulation studies because of the numerous descriptions of modification of DC responses to pathogens by these molecules and because several of them signal through Syk-independent pathways. Amongst them, it would be particularly interesting to investigate in detail the dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), as its ligation has been shown to induce the phosphorylation of serine 276 on the p65 subunit of NF- κ B, leading to the acetylation of this molecule. Strikingly, ManLAM, a cell wall component of mycobacteria that binds DC-SIGN, did not induce p65 translocation to the nucleus on its own, but was able to modify the cytokine response to the whole pathogen when p65 translocation was induced by TLR ligation. Salp15, another DC-SIGN ligand produced by ticks was reported to limit the TLR2/4-induced IL-6, IL-12 and TNF α production [62]. However, DC-SIGN was not the only molecule reported to reduce the cytokine response to TLR ligation. A very similar effect was observed with the mannose receptor (MR) [63]. Thus, as these features of DC-SIGN and MR signalling seem to fit particularly well the effects observed upon LPS and HES co-treatment, we would suggest these CLR s as targets for future studies. Related to this, a good kinase target could be Raf-1, since the DC-SIGN signalling has been shown to be completely dependent on this molecule [62].

The next part of our work focussed on the TLR pathway. As mentioned in the introduction, this pathway is crucial for the recognition of pathogens by the innate immune cells and is likely to detect at least some HES molecules. Since at least 13 TLR s have been discovered, the best way to start the study of the pathway was to alter crucial adapter molecules downstream of the receptors themselves to avoid having to block them one by one. In the case of the TLR s, the task was easier than for the CLR s, as the disruption of the activity of only two molecules, MyD88 and TRIF, was sufficient to block totally the whole pathway. The difficulty, here, was the direct involvement of the pathway in the LPS driven immunomodulation assay. Thus, we could only block one adapter at a time to allow some signalling to induce cytokines, or we could find another TLR-independent way to activate DCs. The experiments on MyD88^{-/-} and TRIF^{-/-} gave us first evidence that HES does not need any TLR to skew the DC inflammation state. After having showed that the CD40

ligation model could be used in our setting, we were able to use MyD88^{-/-}xTRIF^{-/-} BMDCs and confirm the results obtained with the single knockouts. Nevertheless, this experiment was performed only once and the CD40 ligation did not induce a significant up-regulation of the co-stimulatory markers used as read-out. Thus, our statement still needs confirmation.

In addition to these two adapter molecules, we also wanted to characterise the signalling of the HES immunomodulation at the level of cytoplasmic kinases, as we did with Syk for the CLR signalling. Several pathways, mostly involving molecules from the MAPK family could be targeted. Nevertheless, molecules such as extracellular signal-regulated kinase (ERK) seem to regulate DCs survival rather than their activation [64]. Therefore, based on the previously cited papers [21-24], we began this study by investigating the role of PI3K, a kinase crucial for the regulation of cell survival, proliferation and motility that is also involved in the regulation of the immune response through modulation of the NF- κ B activity which is mostly responsible for the early response to pathogen products. In several experiments, different concentrations of the PI3K inhibitor wortmannin were tested. The results indicated no modification of the immunomodulatory properties of HES suggesting that this kinase was unnecessary for the modulation. Thus, the next studies on this subject could focus on the importance of other parallel signalling cascade. Phosphorylation states of different kinases of the MAPK pathways could, for example, be assessed by western blots and compared between populations of DCs exposed to LPS, HES or both products. Besides the classical targets of this pathway, like MEK/ERK or p38, it would be particularly interesting to measure the phosphorylation of Raf-1 according to its importance in the CLR signalling. Alternatively, future work could also consider studying the suppressor of cytokine signalling (SOCS) family members. SOCS2, for example, has been very recently shown to be an important modulator of the *Schistosoma mansoni* induced Th2 response [65].

Next, leaving the DCs aside for a while we tried to reduce the number of potential HES immunomodulators which is now around 370, that is the total number of protein identities found in HES. To do so, we had the opportunity to use already prepared HES size fractions in our immunomodulation assay. We repeatedly showed that a few fractions (mainly 10 and 14) concentrated the immunomodulatory properties and therefore that a fractionation followed by functional assays was an interesting strategy to study the effects of HES on BMDCs. Unfortunately, we were unable to go much further with this approach since both the mass spectrometry and the lectin

binding study did not seem very efficient to select a limited number of targets. Thus, Professor Maizels suggested two fractionation strategies. The first one is sequential and consists in an ion-exchange chromatography on active size fractions to further separate proteins according to their charge. This approach would reduce the costs of mass spectrometry analyses, but would require a large amount and a strong immunomodulatory effect of the size fraction analysed. This should not be a problem for fraction 14, once size fractions will be available in a buffer allowing the ion-exchange step. Otherwise, a checkerboard strategy could be used for fractions with weaker immunomodulatory properties. Both size and charge fractions of total HES could be assessed in parallel functional assays that should provide ranges of mass and charges that concentrate a certain activity. As every single protein in HES is known, it could be possible to select proteins, which match the mass and charge of the previously highlighted ranges. Finally, another simple method to reduce the number of identities in a single fraction is to fractionate more. In this view, HES has already been fractionated in 48 size fractions instead of 24.

Another approach, suggested at the beginning of the project, was to use flow cytometry in order to detect physical interactions between HES and DCs. Since we had no particular target amongst the HES components, we could only use a modified version of the total product that could allow detection with a fluorochrome-associated antibody. Luckily, a small amount of HES had already been biotinylated by James Hewitson and was ready to use. In addition this product turned out to bind BMDCs efficiently and could therefore be used to assess the binding affinity for DCs from various knockout mice.

Hewitson suggested CD24 as a target because he had discovered that HES was binding to B cells through this molecule (unpublished data). Our results confirmed that HES also binds DCs via CD24, but, in contrast to CD24^{-/-} B cells that do not bind HES at all, HES is still able to bind CD24 deficient DCs to some extent. Therefore, we hypothesise that one or more HES components bind at least two receptors on the DCs surface. In terms of functional significance, future work will have to confirm one of the two following hypothesis. Either, as suggested by the IL-12p70 production, HES can fully immunomodulate CD24^{-/-} DCs, or CD24 ligation by HES is contributing to the reduction of inflammatory cytokines, in which case the immunomodulation should be less efficient on CD24^{-/-} DCs. In any case, we have at least showed that CD24 is not crucial for DCs immunomodulation.

Nevertheless, this does not mean that the HES-CD24 interaction has no effect at all. It could, for instance, influence DC maturation, migration, antigen presentation or T-

cell polarisation as the cytokine profile of the DCs does not seem to correlate with the T helper response profile in our experiments. Therefore, there are several other experiments that could be performed with CD24^{-/-} BMDCs, including maturation assays and in vivo transfers, to further investigate the function of the HES interaction with CD24.

Regarding the HES molecules that could bind CD24, Liu et al. [53] strongly emphasize the role of DAMPs. They showed by coimmunoprecipitations that HMGB1, HSP70 or HSP90 bind CD24. Interestingly, the mass spectrometry analysis performed in our lab reveals that HSP70, but not the two other molecules, is a component of HES [66]. However, it is also found in Hex and our results clearly show that Hex does not bind CD24⁺ DCs. Moreover, according to the mass spectrometry HSP70 is more abundant in Hex than in HES. A difference in post-translational modifications between the *H. polygyrus* HSP70 and the host HSP70 could explain the different binding properties of HSP70, but the most probable hypothesis is that HSP70 is so rare in both Hex and HES in comparison to other HES components, that it does not influence the global binding pattern.

The comparison of HES binding to BMDCs, BMDMs and spleen-isolated B cells suggest that the other receptor(s) bound by biotinylated HES is (are) highly expressed on bone marrow-derived macrophages, less expressed on BMDCs and absent from B cells surface. Regarding the C-type lectins, this rules out the DC immunoreceptor (DCIR), which is expressed on B cells [67] and does not argue in favour of the previously suggested target DC-SIGN, which is mainly expressed on DCs. However, the mannose receptor could fit this description as it is only expressed on macrophages and DCs. Moreover, the signalling of this CLR has, to our knowledge, not been shown to be Syk dependent and remains therefore the best potential target for upcoming work. However, this comparison was only qualitative in this setup since DCs and macrophages could not be stained at the same time as BMDMs were grown in 7 days whereas BMDCs needed 10 days of cultures. Therefore, an interesting experiment to do, when enough CD24^{-/-} mice will be available, would be to grow macrophages and DCs from different mice in order to be able to stain both cell types simultaneously and therefore have consistent fluorescence values for each markers. To compare wild type DCs and macrophages one could also consider growing one cell type from a C57BL/6 Ly5.1 background and the other from a Ly5.2 background. This would allow for a direct comparison of both cell types by staining them in the same well. Then, these cell type-dependent HES binding properties could help identify the CD24 ligand in HES.

In conclusion, in this work we characterised the immunomodulatory effects of *H. polygyrus* on GM-CSF grown BMDCs in vitro as well as in vivo and tried to define through which molecular mechanism this modulation occurs. *H. polygyrus* excretory/secretory products showed an amazingly powerful ability to inhibit the LPS- and *P. acnes* extract-induced DCs inflammatory cytokines production and co-stimulatory markers up-regulation even if added in the cell environment hours after the bacterial stimulus. Boiling HES for 20 minutes mainly abrogated this ability, but did not change the T cell response upon adoptive transfer of DCs. This suggests that the in vitro DCs phenotype and cytokine production profile is not sufficient to predict the outcome of the adaptive response in vivo.

Based on Grainger's work, we assessed the role of the TGF β receptor that turned out not to be implicated in the immunomodulatory process. We also investigated the CLR and TLR pathways for their largely described abilities to induce and modulate the response of immune cells to pathogen-associated molecular patterns. TLRs were shown not to be important for the HES immunomodulation of DCs. The roles of two CLRs as well as one downstream kinase were ruled out, but other targets were defined for subsequent studies. Unfortunately, no HES compound could be picked as a target, but strategies were defined to achieve this goal in a near future. Finally, one DC surface receptor was proven to interact with HES and will maybe allow for the isolation of DC-binding HES molecules.

Additional work will be needed to precisely define all the DCs receptors targeted by HES molecules, since CD24 is clearly not the only one. For now, we still have no idea of which HES compounds affect the DCs response. This question could be tackled with the help of HES size and charge fractions that will help to reduce the number of targets in functional assays. As the interaction with CD24 is now confirmed, this property could also be used to characterise at least one HES component interacting with DCs. Different CLRs remain to be studied and a phosphorylation analysis of intra-cellular key molecules from the MAPK, STAT or SOCS pathways could be started as well. The adoptive transfer protocol must also be optimised so that, when inhibition of the HES immunomodulation will be achieved in vitro, the modifying agent could also be tested in vivo. If successful these studies will provide new targets for vaccination against *H. polygyrus* and hopefully other human-infecting helminths. Moreover, HES active compounds could be useful basis for the development of new anti-inflammatory drugs that could be used against autoimmune or allergic diseases.

Appendix

Co-stimulatory markers expression on *MyD88*^{-/-} and *TRIF*^{-/-} DCs

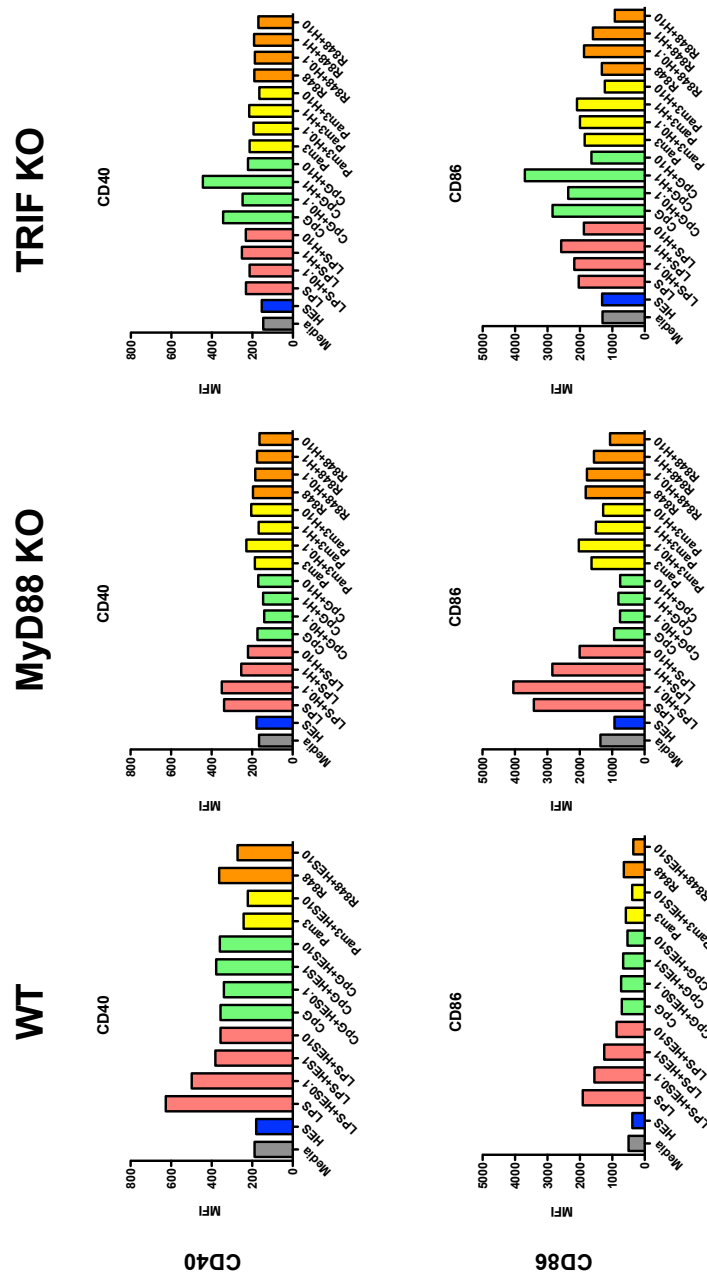


Figure S 1 - Co-stimulatory markers expression analysis on wild type (WT), *MyD88*^{-/-} and *TRIF*^{-/-} BMDCs, Part 1.

Following the assay described in figure 17, triplicate wells were pooled and DCs were stained for CD11c, MHC class II, CD40, CD80 and CD86. Live cells expressing CD11c were gated and the geometric mean of the fluorescence intensity (MFI) of CD40- and CD86-linked fluorophores was plotted. Labels display HES dose in $\mu\text{g/ml}$. Data are representative of two similar experiments.

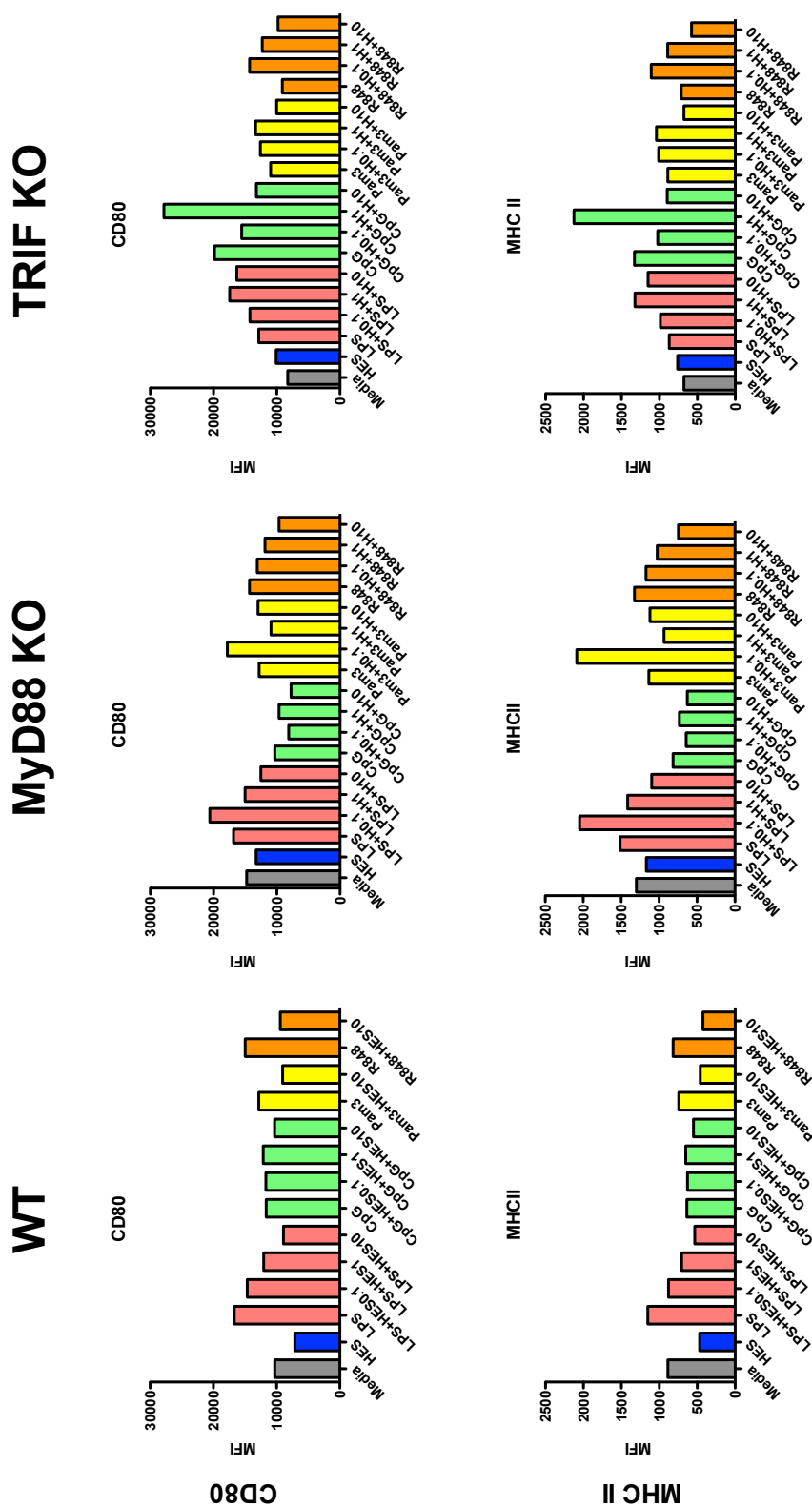


Figure S 2 - Co-stimulatory markers expression analysis on wild type (WT), MyD88^{-/-} and TRIF^{-/-} BMDCs, Part 2.

Following the same analysis as in figure S 1, MFI of CD80 and MHC class II were plotted. Data are representative of two similar experiments.

HES size fractions analysis

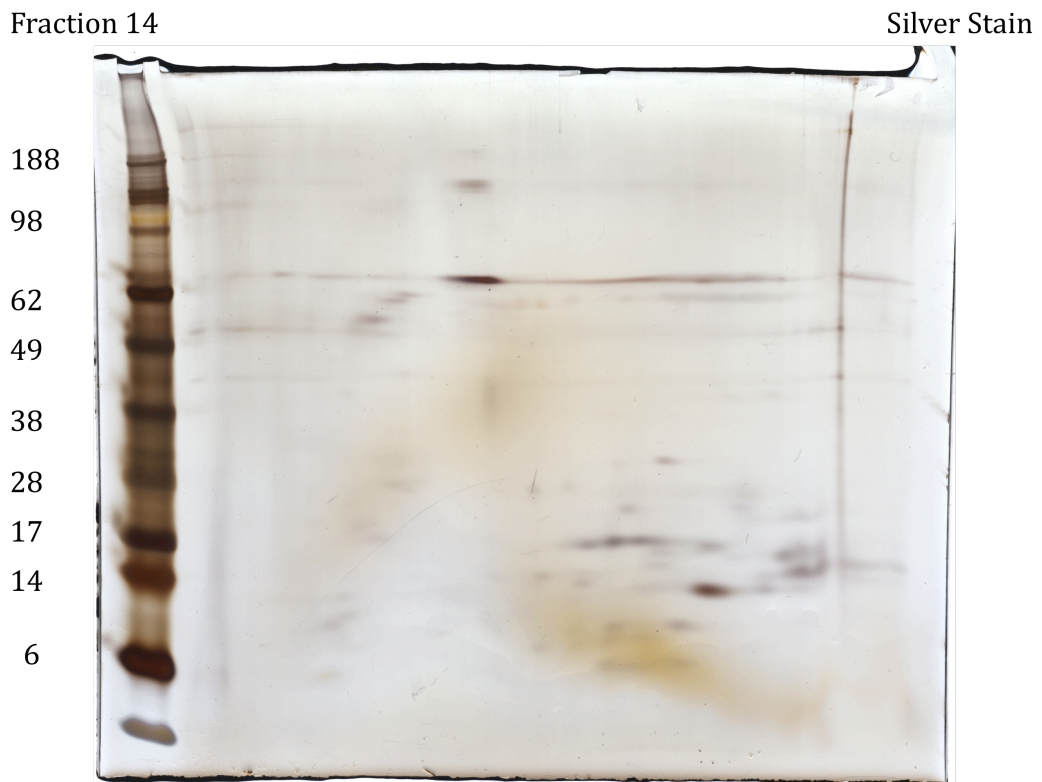


Figure S 3 - Silver staining of a 2D gel of HES size fraction 14
Ladder labels are the apparent protein molecular weights in kDa

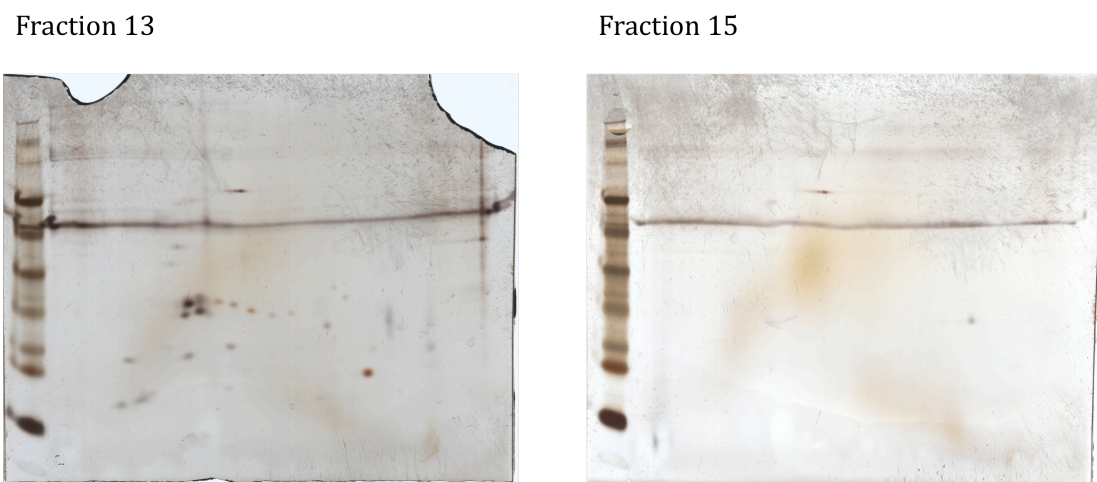


Figure S 4 - Silver staining of 2D gels of HES size fraction 13 and 15

Biotinylated HES/DCs binding analysis

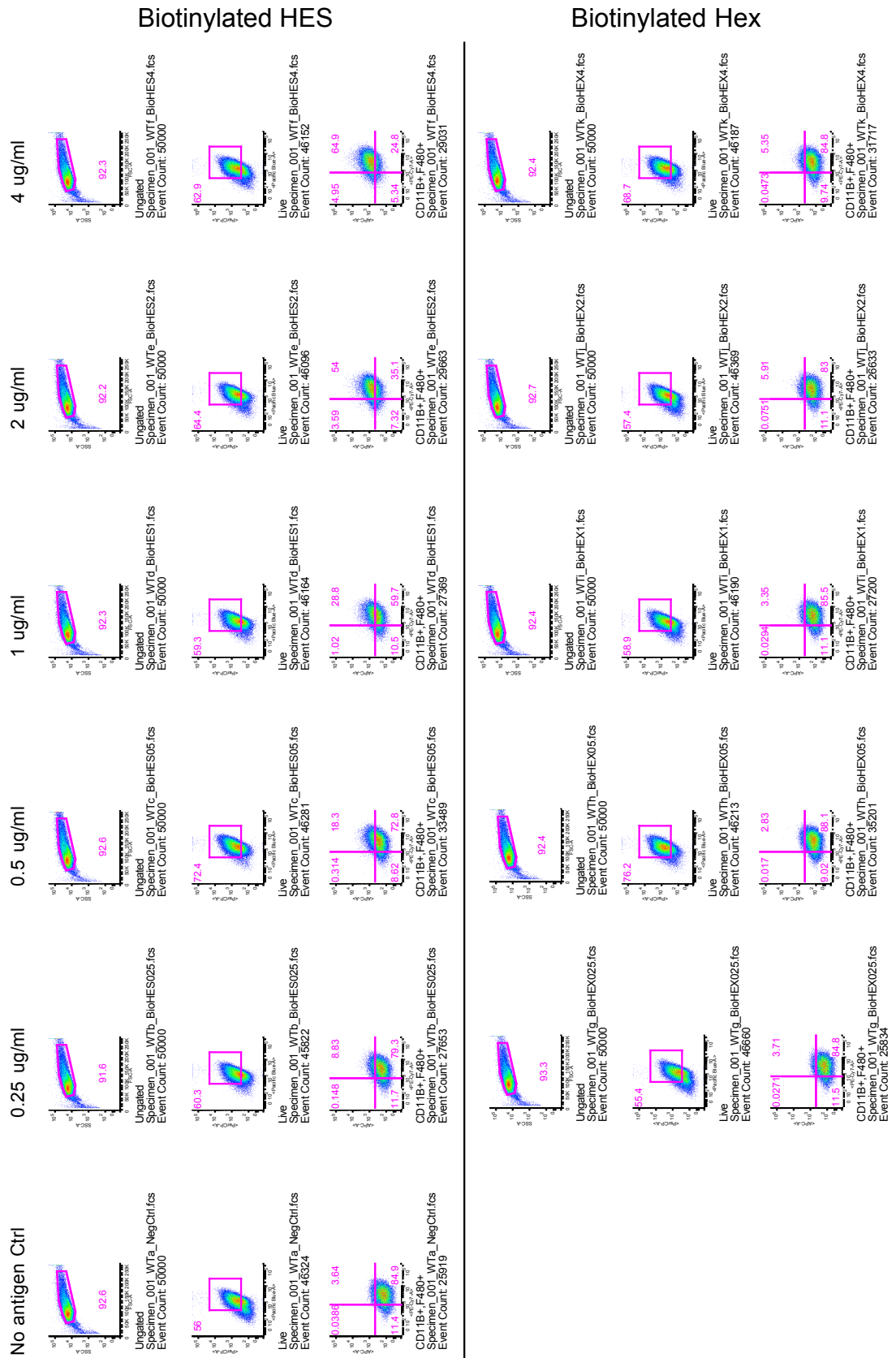


Figure S 5 – Flow cytometry analysis of wild type BMDMs.

In a first step, wild type BMDMs were stained for CD11b, CD11c, F4/80, MHC class II, CD24 and different concentrations of biotinylated HES or biotinylated Hex. A secondary stain with streptavidin-APC allowed for the detection of HES or Hex binding. Live cells were gated according to their forward and side scatters (first and fourth rows of the board), then their expression of CD11b and F4/80 (second and fifth rows) and, finally, the expression of CD24 and biotinylated HES (third row) or Hex (sixth row) were plotted on the x and y axis respectively. The first column shows the negative control that was not stained for HES or Hex. The second to sixth columns were respectively stained with 0.25, 0.5, 1, 2 or 4 μ g/ml biotinylated HES or Hex. Gates and percentages of parent cell population gated in are displayed.

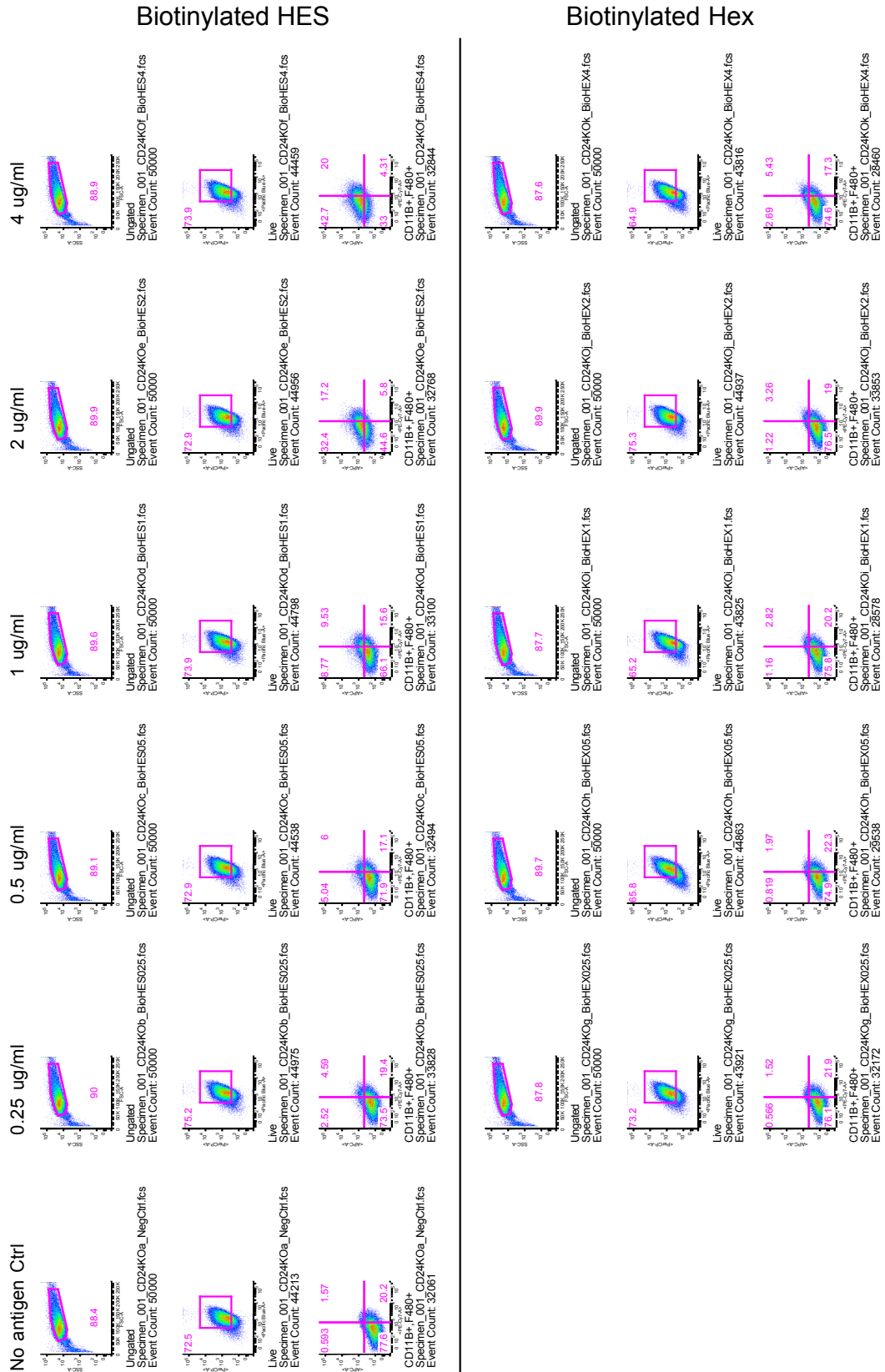


Figure S 6 - Flow cytometry analysis of CD24^{-/-} BMDMs

CD24^{-/-} BMDMs were stained and analysed following the same protocol as in figure S 4. Gates and percentages of parent cell population gated in are displayed.

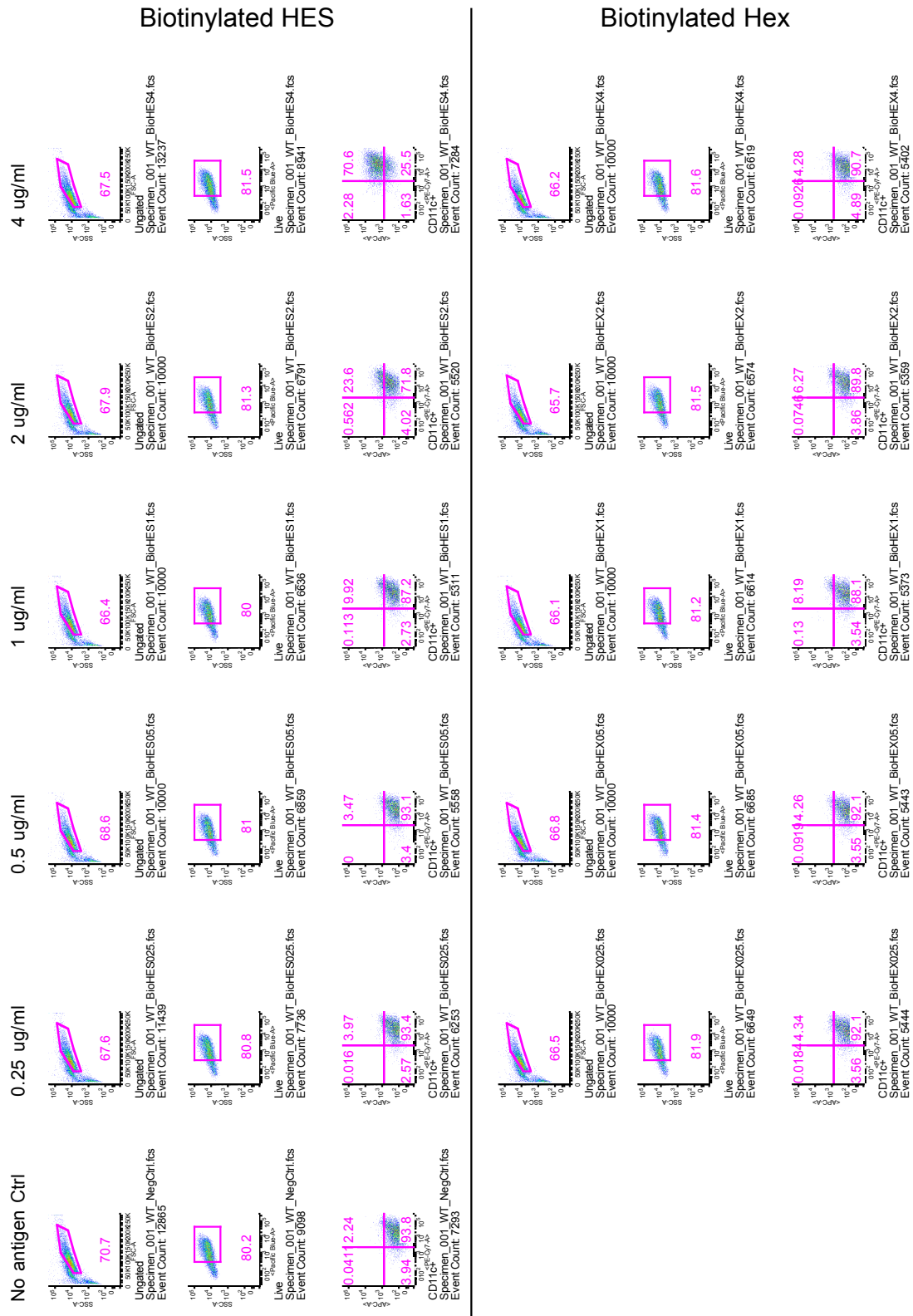


Figure S 7 - Flow cytometry analysis of wild type BMDCs

Wild type BMDCs were stained for CD11c, MHC class II, CD40, CD86, CD24 and different concentrations of biotinylated HES or biotinylated Hex. The analysis was the same as for macrophages (Fig. S 3 and 4), except that live cells were gated for their expression of CD11c instead of CD11b and F4/80. Gates and percentages of parent cell population gated in are displayed.

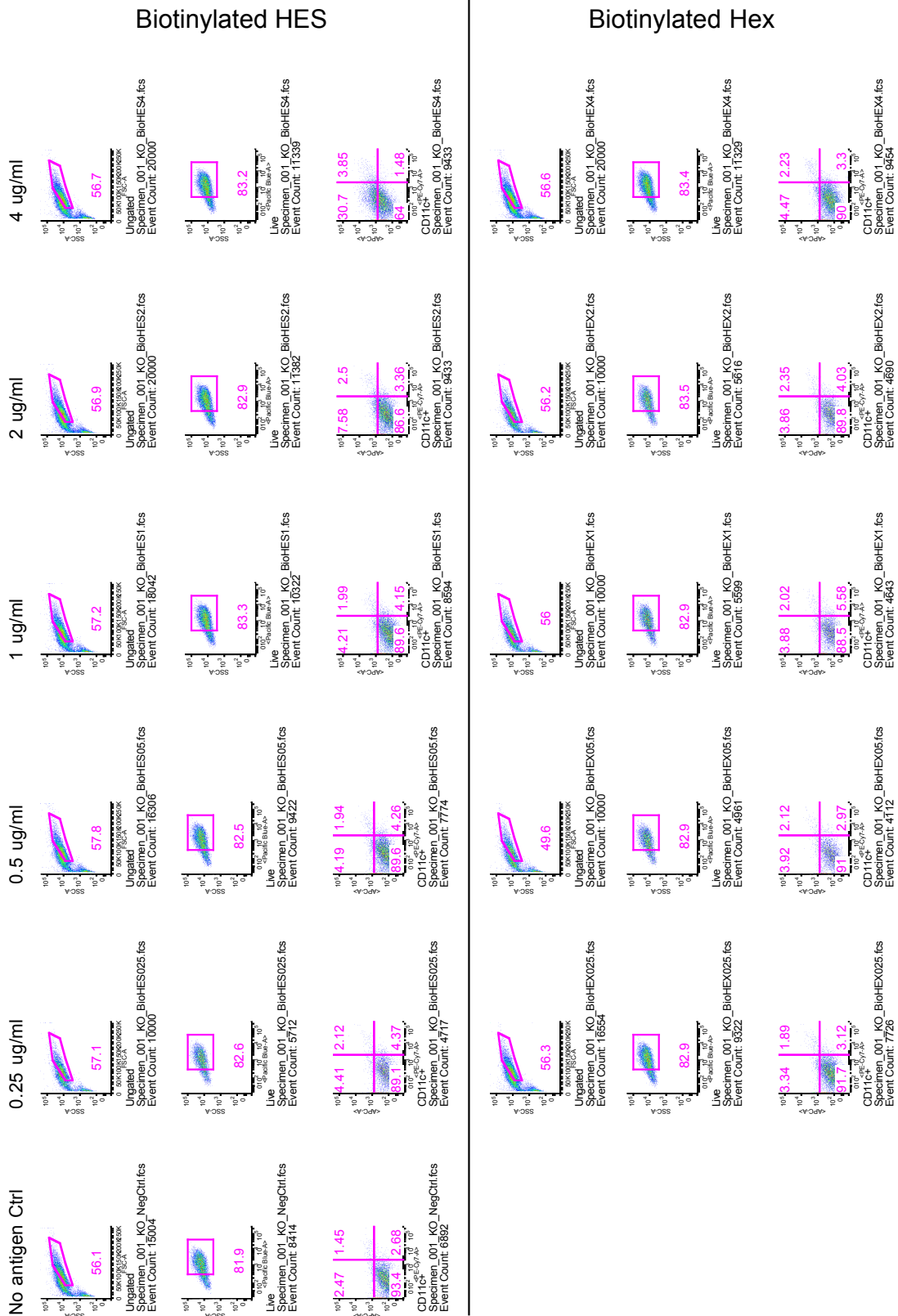


Figure S 8 - Flow cytometry analysis of CD24^{-/-} BMDCs

CD24^{-/-} BMDCs were stained and analysed following the same protocol as in figure S 5. Gates and percentages of parent cell population gated in are displayed.

CLR and TLR signalling pathways and inhibitors used in this study

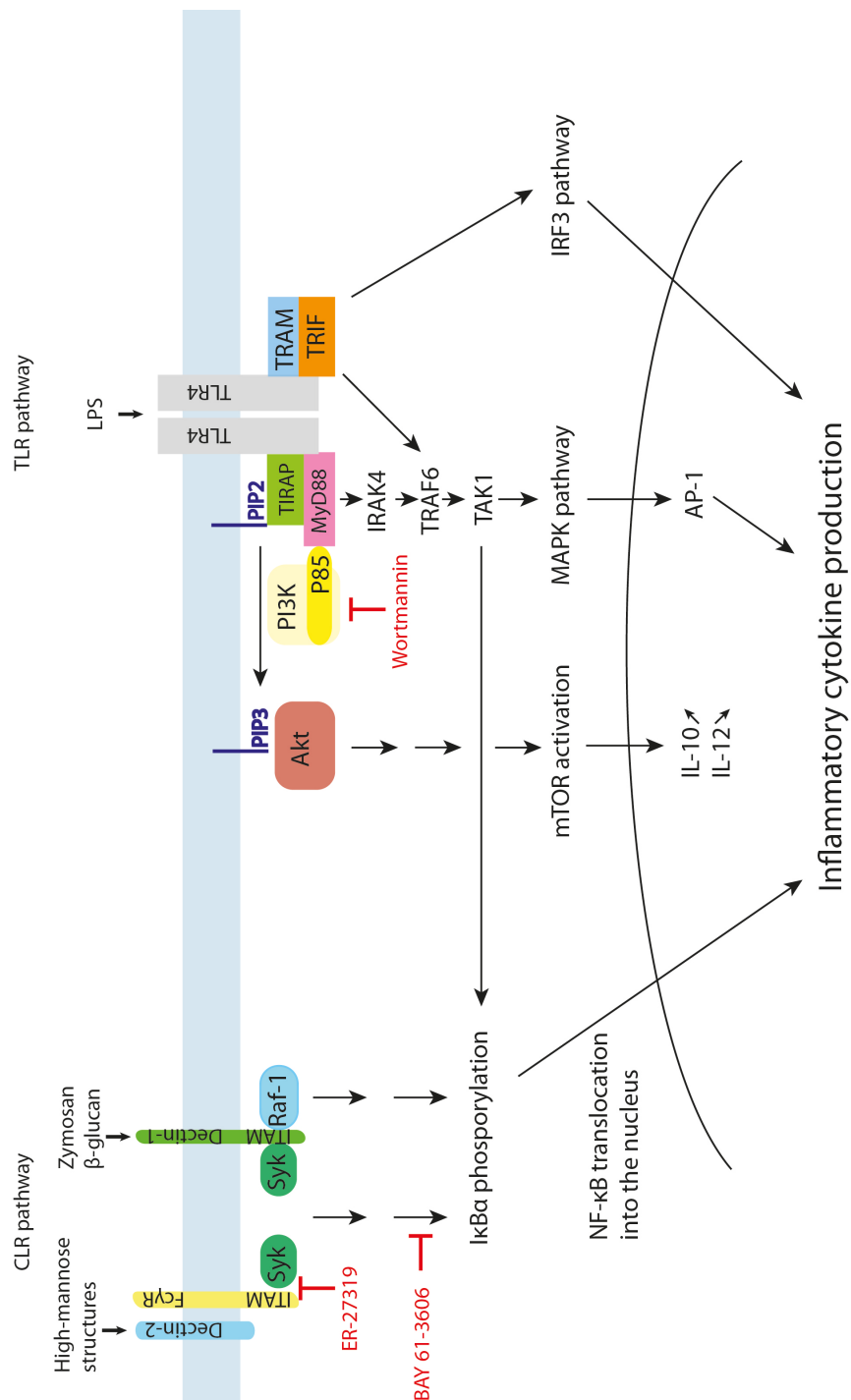


Figure S 9 – Important molecules and inhibitors of the CLR and TLR pathways considered in this work.

This figure is not exhaustive and its purpose is only to ease the understanding of a few paragraphs of this work. This schematic compiles information from papers cited in these paragraphs, but also from a review by Kawai & Akira [68]. Inhibitors used during the project are highlighted in red.

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