Effects of aqueous extracts from bogbean and yarrow on human dendritic cells and their ability to activate allogeneic $CD4^+$ T cells *in vitro*

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Thesis for the degree of Master of Science
University of Iceland
Faculty of Medicine
School of Health Sciences

HÁSKÓLI ÍSLANDS

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Áhrif vatnsútdrátta af horblöðku og vallhumli á þroska angafrumna og getu þeirra til að ræsa ósamgena $CD4^+$ T frumur *in vitro*

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Ágrip

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Plönturnar horblaðka, blágresi, mjaðjurt og vallhumall hafa verið notaðar í alþýðulækningum í aldaraðir og taldar hafa góð áhrif á ýmsa bólgusjúkdóma, s.s. gigt. Nokkur efnasambönd hafa verið einangruð úr þessum plöntum en aðeins fáar rannsóknir hafa verið gerðar til að kanna áhrif þeirra á ónæmiskerfið og engar upplýsingar eru til um áhrif þessara plantna á angafrumur. Angafrumur eru öflugustu sýnifrumur ónæmiskerfisins og eru mikilvægur hlekkur á milli ósérhæfða og sérhæfða ónæmiskerfisins. Hlutverk þeirra er að taka upp vaka og sýna óreyndum T frumum sem ræsast og bregðast við með sérhæfðu ónæmissvari.

Markmið verkefnisins var að kanna hvort etanól- eða vatnsútdrættir af plöntum sem notaðar hafa verið í alþýðulækningum við gigt hefðu áhrif á þroska angafrumna og getu þeirra til að ræsa ósamgena $CD4^+$ T frumur *in vitro*.

Útdrættirnir voru útbúnir með því að setja þurrkaðar plöntur í etanól eða heitt vatn. Angafrumur voru sérhæfðar úr einkjörnungum úr mönnum og síðan þroskaðaðar með eða án útdrátta af horblöðku, blágresi, mjaðjurt eða vallhumli. Áhrif útdráttanna á þroska angafrumnanna voru metin með að því að mæla seytingu þeirra á boðefnunum IL-6, IL-10, IL-12p40 og IL-23 með ELISA aðferð og tjáningu yfirborðsameindanna CD14, CD86, CCR7, DC-SIGN og HLA-DR með frumuflæðisjá. Þar að auki voru angafrumur þroskaðar með vatnsútdráttum af vallhumli eða horblöðku samræktaðar með ósamgena $CD4^+$ T frumum. Könnuð voru áhrif þeirra á ræsingu T fruma með því að mæla tjáningu þeirra á CD4, CD25 og ICAM-1 með fumuflæðisjá, seytingu IL-4, IL-10, IL-17 og IFN-γ með ELISA aðferð og frumufjölgun með innlimun þrívetna týmidíns.

Angafrumur þroskaðar með vatnsútdrætti af vallhumli seyttu minna af IL-12p40 og meira af IL-10 samanborið við angafrumur ræktaðar án útdráttar og hjá angafrumum sem voru þroskaðar með vatnsútdrætti af horblöðku var tilhneiging til minni seytingar á IL-12p40 samanborið við angafrumur þroskaðar án útdráttar. Angafrumur þroskaðar í návist þessara beggja vatnsútdrátta sýndu lækkun á IL-12p40/IL-10 boðefnahlutfalli. Angafrumur þroskaðar með vatnsútdráttum af horblöðku eða vallhumli höfðu engin áhrif á seytingu IL-6 og IL-23. Ósamgena $CD4^+$ T frumur sem voru samræktaðar með angafrumum þroskuðum með vatnsútdrætti af horblöðku seyttu minna af IFN-γ og IL-17 í samanburði við $CD4^+$ T frumur samræktaðar með angafrumum sem voru þroskaðar án útdráttar. Angafrumur þroskaðar í návist vatnsútdráttar af vallhumli höfðu sömu áhrif á IL-17 seytingu en engin áhrif á IFN-γ seytingu ósamgena $CD4^+$ T frumna eftir samræktun. Etanólútdrættir af öllum plöntunum og vatnsútdrættir af blágresi og mjaðjurt höfðu engin áhrif á þroska angafrumnanna.

Niðurstöður rannsóknarinnar benda til þess að vatnsútdrættir af horblöðku og vallhumli leiði til ónæmisdempandi svipgerðar hjá angafrumum sem dregur úr getu þeirra til að ræsa Th17 frumusvar hjá ósamgena $CD4^+$ T frumum og einnig minnkar útdráttur af horblöðku getu angafrumna til að ræsa Th1 frumusvar. Þessar niðurstöður sýna að vatnsútdrættir af horblöðku og vallhumli innihalda efni sem geta hugsanleg dregið úr sjálfsofnæmissjúkdómum þar sem Th1 og/eða Th17 frumur eru ráðandi með því að hafa áhrif á angafrumur og samspil þeirra við $CD4^+$ T frumur. Því er mikilvægt að einangra lífvirka efnið/efnin í vatnsútdráttum af horblöðku og vallhumli með lífvirknileiddri einangrun og skoða áhrif þess nánar *in vivo,* t.d. á liðagigt í rottum.

Abstract

Effects of aqueous extracts from bogbean and yarrow on human dendritic cells and their ability to activate allogeneic $CD4^+$ T cells *in vitro*

The plants bogbean, geranium, meadowsweet and yarrow have been used in traditional medicine for centuries to cure various inflammatory diseases. Several compounds have been isolated from these plants, but few of them have been tested for their effects on the immune system and no data exist on the effects of these plants on dendritic cells (DCs). DCs are the most potent antigen-presenting cells of the immune system and act as a link between the innate and the adaptive immune system. Their responsibility is to take up and present antigens to naïve T cells, which in response activate a specialized immune response.

The aim of this study was to determine whether ethanol or aqueous extracts of plants, which have been used in traditional medicine to ameliorate arthritis, affect stimulation of human DCs *in vitro* and their ability to activate allogeneic $CD4^+$ T cells.

Extracts were prepared by either kinetic maceration of dried plant material in ethanol or by decoction of the plant material in water. Human monocyte-derived DCs were matured in the absence or presence of the extracts from bogbean, geranium, meadowsweet or yarrow. The effects of the extracts on DC maturation were assessed by measuring their secretion of L-6, IL-10, IL-12p40 and IL-23 by ELISA and expression of CD14, CD86, CCR7, DC-SIGN and HLA-DR by flow cytometry. In addition, DCs matured in the presence of aqueous extracts from bogbean or yarrow were co-cultured with allogeneic $CD4^+$ T cells and their effects on activation of the T cells analysed by measuring expression of CD4, CD25 and ICAM-1 by flow cytometry, secretion of IL-4, IL-10, IL-17 and IFN-γ by ELISA, and cell proliferation by thymidine incorporation.

DCs matured in the presence of aqueous extract from yarrow secreted less IL-12p40 and more IL-10 than DCs stimulated without extracts and when DCs were matured in the presence of aqueous extract from bogbean there was a tendency towards reduced IL-12p40 secretion compared with DCs matured without the extracts. Both extracts led to a reduction in the ratio of IL-12p40/IL-10 secreted by the DCs. Maturing DCs in the presence of aqueous extracts from bogbean and yarrow had no effect on IL-6 or IL-23 secretion. Allogeneic $CD4^+$ T cells that were co-cultured with DCs that had been matured in the presence of aqueous extract from bogbean secreted less IFN-γ and IL-17 than $CD4^+$ T cells co-cultured with DCs that had been matured without an extract. DCs that had been matured with aqueous extracts from yarrow had the same effect on IL-17 secretion but did not affected IFN-γ secretion by allogeneic $CD4^+$ T cells after co-culture. Ethanol extracts from all the plants and aqueous extracts from geranium and meadowsweet did not affect maturation of the DCs.

The results from the study indicate that aqueous extracts from bogbean and yarrow induce a tolerogenic-like phenotype of DCs which have reduced capacity to induce Th17 stimulation of allogeneic $CD4^+$ T cells. In addition, they indicate that bogbean extract can reduced the capacity of DCs to induce a Th1 response. These results show that aqueous extracts from bogbean and yarrow contain compound(s) that may possibly reduce Th1- and/or Th17-mediated autoimmune responses by affecting DCs and their interaction with $CD4^+$ T cells. The active compound(s) in the aqueous extracts from bogbean and yarrow need to be identified, using bioguided isolation, and their effects determined *in vivo*, e.g. in a rat model of arthritis.

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ANOVA	Analysis of variance
APS	Antigen presenting cell
B-DC	Dendritic cells matured with bogbean extract
BSA	Bovine serum albumin
CPM	Counts per minute
DCs	Dendritic cells
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorter
GM-CSF	Granulocyte macrophage–colony-stimulating factor
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
imDCs	Immature dendritic cells
LPS	Lipopolysaccharide
MACS	Magnetic activated cell sorting
MC	Monocyte
mDC	Mature dendritic cells (matured without plants extract)
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
NK-cells	Nature killer cells
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PRRs	Pattern recognition receptors
RT	Room temperature
SEM	Standard error of the mean
SI	Secretion index
TNF	Tumour necrosis factor
TLR	Toll-like receptor
Treg	Regulatory T cell
VD3	Vitamin D3
Y-DC	Dendritic cells matured with yarrow extract

1 Introduction

1.1 The immune system

The immune system is a network of proteins, cells, tissues and organs that work together to defend the body against infections. It identifies and destroys pathogens and tumour cells. It also clears the body of foreign substances and dead cells. The key to a healthy immune system is the distinguishing between the body's own molecules and foreign molecules, recognized as self and non-self. This ability is necessary to protect the organism from invading pathogens and to eliminate modified or altered cells (e.g. malignant cells). In addition, the immune system is capable of generating immunological memory against an infectious agent and prevent repeated infections (Murphy *et al.*, 2008). The immune system is divided into innate and adaptive immunity and both these systems are necessary to maintain protective immunity of the individual (Fearon *et al.*, 1996).

All cells of the immune system arise from pluripotent hematopoietic stem cells in the bone marrow. The stem cells divide to form precursors for the two lineages of immune cells, the myeloid and the lymphoid progenitors and eventually form specialized cells with a clearly defined purpose. The innate immune system is the first line of defence against infection and comprises several distinct components that defend the host from the pathogen. If invasive microorganisms manage to cross an epithelial barrier the innate system responds immediately by tissue macrophages equipped with surface receptors that can bind and phagocytose many different types of pathogens and secrete cytokines and chemokines. Then neutrophils, the most potent phagocytes, leave the blood vessels and join macrophages at the site of infection. Dendritic cells (DCs) are another type of phagocytic cells present in tissue. They are antigen-presenting cells (APC) that are able to initiate an adaptive immune response (see section 1.2). The complement system consists of plasma proteins that are able to opsonize pathogens which facilitates their recognition and engulfment by phagocytic cells. It can also produce a local inflammatory response that defends against (initial) infection. Natural kill cells (NK cells), which are potent killers of virus-infected (and some tumour cells) also play a role in the innate immune response. The induction of an adaptive immune response begins when a pathogen is ingested by an immature DC in the infected tissue. It takes longer time to activate the adaptive immune system but it gives a more systematic and stronger immune response than the innate system. The cells of the adaptive immune system have the ability to create immunological memory and are ready to respond rapidly and effectively to the same pathogen if it is encountered another time.

The main cells of the adaptive immune system are two types of lymphocytes, the B and the T cells. They recognise foreign antigens through specific receptors. B cells mature in bone marrow but T cells finish their development in thymus. The adaptive immune system can be divided into the humoral and the cellular immune system. B cells are involved in the humoral immune response, whereas T cells are involved in cell-mediated immune response. B cells most often require help from T cells to become activated and the activated B cells then differentiate into antibody-secreting plasma cells and memory

B cells. Activation of T cells results in several types of effector T cells and memory T cells (see section 1.1.1) (Murphy *et al.*, 2008).

1.1.1 The T cells

While T cells undergo development in the thymus, the immature cells that recognize self major histocompatibility complex (MHC) receive survival signals (positive selection) and those that interact strongly with self antigens are removed from the repertoire (negative selection). The few T cells that survive selection mature and leave the thymus as naïve but mature T cells. They then circulate between the bloodstream and lymphoid tissues, where they may encounter APCs. APCs present antigens to the naïve T cells in connection with the MHC molecules. There are two types of MHC molecules, class I and class II. When naïve T cells recognize specific peptides expressed on MHC molecules they become activated. Activation leads to clonal expansion and differentiation and gives rise to a clone of effector T cells of identical antigen specificity. These effector T cells are attracted to the sites of infection (Murphy*, et al.*, 2008; O'Neill *et al.*, 2004). T cells fall into two major classes, cells that express the CD4 and cells that express the CD8 co-receptors. $CD8^+$ T cells recognize MHC class I molecules and activated $CD8^+$ T cells, cytotoxic T cells, are specialized to kill tumour and virus-infected cells. $CD4^+$ T cells recognize MHC class II molecules and activated $CD4^+$ T cells differentiate into a number of different effector T cells with a variety of functions. The main functional subsets of $CD4^+$ effector T cells are Th1, Th2, Th17 and regulatory T cells (Murphy*, et al.*, 2008; Wan *et al.*, 2009; Zhu *et al.*, 2008).

Th1 cells play a role in activation of infected macrophages, destruction of pathogens that persist in macrophage vesicles, providing help to B cells for production of antibodies directed at extracellular pathogens and induction of class switching. Th2 cells stimulate production of antibodies by B cells, especially IgE antibodies. Th17 cells play a role early in the adaptive immune response. They activate multiple cell types, e.g. fibroblasts and epithelial cells, to secrete soluble mediators such as chemokines that can recruit neutrophils to sites of infection where they play an important role in protection against extracellular pathogens and establishment of chronic inflammation (Murphy*, et al.*, 2008; Pulendran *et al.*, 2010; Romagnani, 2008; Wan*, et al.*, 2009). Regulatory T cells tend to suppress the adaptive immune responses from becoming uncontrolled and prevent autoimmunity.

In addition to providing effector T cells, the primary T cell response also generates memory T cells, which are long-lived cells that give an accelerated response when they encounter the same antigen that activated them initially.

1.2 The dendritic cells

DCs play an important role as a bridge between the innate and the adaptive immune systems (Palucka *et al.*, 1999; Steinman, 1991) as initiators and modulators of the immune response (Banchereau, 1998; Moser, 2000). DCs perform two distinct functions, on one hand they act as sentinels for dangerous antigens in peripheral tissues and on the other hand they are specific APCs for naïve T cells in the lymphoid organs (Moser, 2000). APCs are the only cells in the body, apart from the thymic cortical epithelium, that express MHC II molecules. MHC II molecules deliver peptides originating in the vesicular system to the cell surface, whereas MHC I molecules deliver peptides originating in the cytosol to the cell surface. DCs are the most potent APCs but macrophages and B cells can also act as APCs (Banchereau *et al.*, 1998; Murphy*, et al.*, 2008). DCs were first observed in 1868 as Langerhans cells of the epidermis and they were identified within the spleen in 1973. A low frequency of DCs in the blood (1-2% of the total leukocytes) makes their isolation problematic and slowed the progress of their characterization. For the past decade, advances in *in vitro* methods have provided a reliable source of DCs for functional and development studies. It has become clear that DCs are important for the immune system and are important in studies of the immune system and its role in various diseases (Palucka*, et al.*, 1999; Sato *et al.*, 2007).

1.2.1 DC differentiation and subtypes

DCs arise from pluripotent CD34+ hematopoietic stem cell and mature along both myeloid and lymphoid lineages in the bone marrow (Murphy*, et al.*, 2008). The two main populations of DCs; from myeloid progenitors called conventional DCs or myeloid DCs, and from lymphoid progenitors called plasmacytoid DCs, differ in many ways, including their distribution, cytokine production and growth requirements (Murphy*, et al.*, 2008; O'Neill*, et al.*, 2004). Conventional DCs have a dendritic form and exhibit DC functions in steady state, but plasmacytoid DCs require further development to acquire a dendritic form and full DC function (figure 1) (Shortman *et al.*, 2007).

The complexity of DCs is enhanced by the fact that there is no single surface molecule known to be only expressed by DCs, they can be found at different developmental stages and at different locations in the body (Palucka*, et al.*, 1999). The combination of several markers, however, can be used to define a DC subpopulation and its stage of maturation (Murphy*, et al.*, 2008; Palucka*, et al.*, 1999).

Conventional DCs are found in many tissues, secondary lymphoid organs and blood (Uneo *et al.*, 2007). Myeloid progenitor cells differentiate into two types of conventional DCs, CD11c+CD1a+ Langerhans cell precursors, and CD11c+CD1a- interstitial DC precursors in blood (Lange *et al.*, 2007; Liu, 2001; Sato, 2007). The Langerhans cell precursors migrate into the skin epidermis and become Langerhans cells, which express the C-type lectin Langerin, have unique intracellular organelles called Birbeck granules, and are found in the epidermis and oral, respiratory, and genital mucosa (Lange*, et al.*, 2007; Liu, 2001; O'Neill*, et al.*, 2004; Uneo*, et al.*, 2007). The interstitial DC precursors migrate into

the skin dermis and other tissues to become interstitial DCs, that express DC-SIGN (CD-209), CD11b, factor XIIIa and CD14 (Lange*, et al.*, 2007; Liu, 2001; Uneo*, et al.*, 2007). Conventional DCs display the classical properties of DCs, capturing antigen in the peripheral tissues, migrating to secondary lymphoid organs and presenting these antigens to naïve T cells and activating them (Lange*, et al.*, 2007; Murphy*, et al.*, 2008).

There is only one type of plasmacytoid DCs; they are CD11c-, express BDCA-2, a C-type lectin and are found in blood and lymphoid tissues. They are round, non-dendritic, relatively long-lived and circulating cells (Murphy*, et al.*, 2008; Shortman*, et al.*, 2007; Uneo*, et al.*, 2007). Plasmacytoid DCs express high levels of MHC II molecules and secrete large amounts of type I interferons (IFNα and IFNβ) after stimulation by viral or other microbial infections (figure 1). They are important in innate anti-viral immunity and can activate anti-tumour and anti-viral antigen responses but their potential as immunotherapeutic adjuvants is largely unexplored because they are difficult to obtain in large quantities (Lange*, et al.*, 2007; O'Neill*, et al.*, 2004; Shortman*, et al.*, 2007).

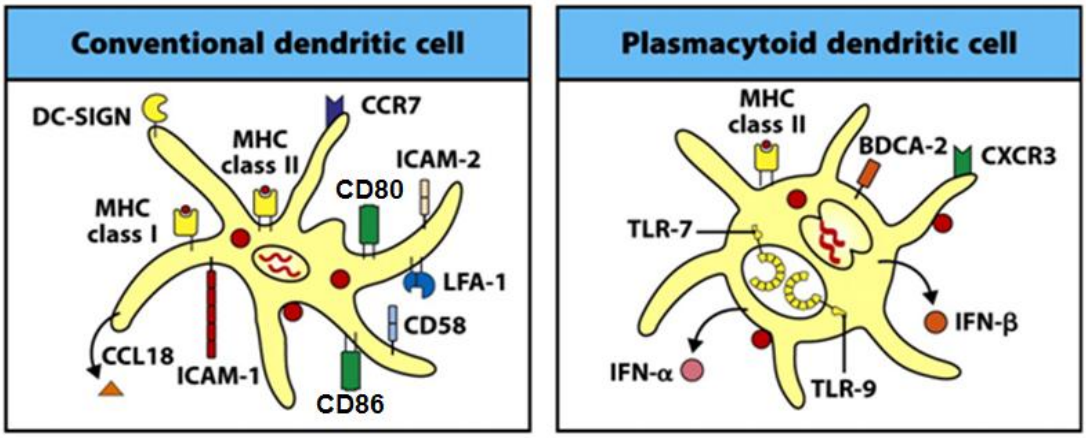

Figure 1. The difference between mature conventional dendritic cell and plasmacytoid dendritic cell.
There are several subsets of conventional dendritic cells which express various cell-surface molecules. The figure shows the main cell-surface molecules expressed by matured conventional dendritic cell and plasmacytoid dendritic cell. Figure adapted from (Murphy*, et al.*, 2008).

1.2.2 Antigen uptake and DC maturation

DCs play a key role in initiating specific immune responses and they can induce such contrasting states as active immune responsiveness or immunological tolerance (Palucka*, et al.*, 1999; Uneo*, et al.*, 2007). Immature DCs (imDCs) reside under most surface epithelia and in most solid organs, where they are taking up particles and liquid from their environment. They capture bacteria, viruses, dead or dying cells, proteins, and immune complexes through phagocytosis, endocytosis, and pinocytosis. When imDCs become aware of foreign molecules they have to determine whether they are safe or dangerous molecules. They also have ability to distinguish self and non-self molecules and they participate in maintaining tolerance against self antigens (Murphy*, et al.*, 2008). ImDCs have an array of cell surface receptors for antigen uptake, many of which also function in signalling or cell-cell

interactions (Murphy, *et al.*, 2008; O'Neill, *et al.*, 2004). ImDCs have FcγRI and FcγRII and complement receptors that take up opsonized antigens (Banchereau, *et al.*, 1998; Murphy, *et al.*, 2008; O'Neill, *et al.*, 2004). ImDCs also express various pattern-recognition receptors (PRRs) that recognize signature microbial molecules considered to be essential components for the survival of the pathogens and are known as pathogen-associated molecular patterns (PAMPs). PAMPs can be lipopolysaccharides (LPS), peptidoglycans, lipoproteins, flagellin, DNA, RNA and more. The best characterized class of PRRs are the Toll-like receptors (TLRs) (Kumar *et al.*, 2009; Lee *et al.*, 2007; Murphy, *et al.*, 2008). TLRs do not participate in phagocytosis but are thought to be the most important PRRs on imDCs as they activate them, which subsequently leads to activation of adaptive immunity (Murphy, *et al.*, 2008). The mammalian genome includes 12 TLRs and many of the ligands for the TLRs have now been identified (Akira *et al.*, 2006; Joffre *et al.*, 2009; Kumar *et al.*, 2009). The TLR family members can be roughly divided into those that recognize viruses and those that recognize bacterial and protozoan pathogens based on their subcellular locations (Kumar, *et al.*, 2009; Lee, *et al.*, 2007). Some PRRs are highly phagocytic, such as the C-type lectins, DEC 205 (CD205) and DC-SIGN, mannose receptors and scavenger receptors (Banchereau, *et al.*, 1998; Lee, *et al.*, 2007; Murphy, *et al.*, 2008). DCs process captured proteins into peptides that are loaded onto MHC II molecules and these peptide MHC complexes are transported to the cell surface for recognition by antigen-specific $CD4^+$ T cells. An alternative pathway also exists whereby DCs process exogenous antigens onto MHC I molecules. This pathway, called cross presentation, permits DCs to elicit CD8 as well as CD4 T cell responses to exogenous antigens such as apoptotic or necrotic tumour cells, virus-infected cells and immune complexes, allowing DCs to stimulate anti-viral responses by $CD8^+$ T cells (Banchereau, 1998; Murphy, *et al.*, 2008; O'Neill, *et al.*, 2004).

When imDCs become activated they downregulate chemokine receptors that maintain DCs in the tissues (e.g. CCR6) and upregulate CCR7 (CD197), which enhances their ability to migrate from the peripheral tissues to the draining lymph nodes. CCR7 binds the chemokines CCL19 and CCL21 that are highly expressed in lymphoid tissues and direct the $CCR7^+$ DCs to the draining lymph nodes (Lee, *et al.*, 2007; Murphy, *et al.*, 2008; Palucka, *et al.*, 1999; Sato, *et al.*, 2007). As a result of the activation of the DCs, they also upregulate their expression of the MCH molecules and the costimulatory molecules CD80 (B7-1), CD86 (B7-2) and CD40. They also express very high levels of several adhesion molecules, such as ICAM-1 (CD54), ICAM-2 (CD102), DC-SIGN and LFA-3 (CD58) (figure 1) (Murphy, *et al.*, 2008; Palucka, *et al.*, 1999). Mature DCs secrete CCL18 (DC-CK), which specifically attracts naïve T cells to the lymph nodes. When DCs become activated and migrate to the lymph nodes they lose their capacity to take up antigens but become potent APCs, competent to sustain the expansion and differentiation of antigen-specific naïve T cells into appropriate effector cells, thereby initiating primary immune response (Joffre *et al.*, 2009; Lee, *et al.*, 2007; Sato, *et al.*, 2007).

1.2.3 Activation and differentiation of T cells

DCs deliver three kinds of signals for clonal expansion and differentiation of naïve T cells. Signal 1 derives from the interaction of a specific peptide MHC complex on the DCs with the T cell receptor on the naïve T cells. Effective activation of naïve T cells requires signal 2, the costimulatory signal, which has to be delivered by the same DC delivering signal 1. The best known costimulatory molecules are CD80 and CD86 which bind to CD28 on the T cells (figure 2) (Joffre*, et al.*, 2009; Lee*, et al.*, 2007; Palucka*, et al.*, 1999). After receiving signal 1 and 2 the activated T cells express a high-affinity IL-2 receptor and secrete IL-2, which induces the T cells to proliferate. T cells whose antigen receptors are ligated in the absence of costimulatory signals fail to make IL-2 and instead become anergic or die. T cells also start to express CD40L which binds to the CD40 on DCs. In addition to providing supplementary costimulatory signals to the T cells, this leads to increased DC survival and upregulation of CD80 and CD86 expression that promotes T cell proliferation (Banchereau*, et al.*, 1998; Kapsenberg, 2003; Murphy*, et al.*, 2008). Signal 3, provided by the cytokine milieu surrounding the T cells, acts on the T cells to promote their differentiation into effector T cells. These cytokines are mainly secreted by the DCs, which can secrete many cytokines, such as IL-1, IL-6, IL-12, IL-23, IL-10 and TGFβ (figure 2) (Joffre*, et al.*, 2009; Lange*, et al.*, 2007). Important factors, other than signals delivered by DCs that drive primary immune responses are concentration of antigen in the microenvironment, concentration of cytokines and other soluble factors present in the fluid phase in the vicinity of the DC-T cell interface and, of course, the genetics of the host that may limit how the interacting cells may respond (Lipscormb *et al.*, 2002; Uneo*, et al.*, 2007). At the same time as providing effector T cells, activation of naïve T cells also generates memory T cells, which are long-lived cells that give an accelerated response to a further encounter with the same antigen (Banchereau, 1998; Murphy*, et al.*, 2008; O'Neill*, et al.*, 2004; Weaver *et al.*, 2006). DCs can direct differentiation of $CD4^+$ T cells into a different effector T cells, the main functional subsets are, as previously mentioned, Th1, Th2, Th17 and the regulatory T cells (Treg) (figure 2) (Murphy*, et al.*, 2008; Zhu*, et al.*, 2008). In contrast, $CD8^+$ effector T cells take on a uniform cytotoxic phenotype (Lee*, et al.*, 2007; Murphy*, et al.*, 2008).

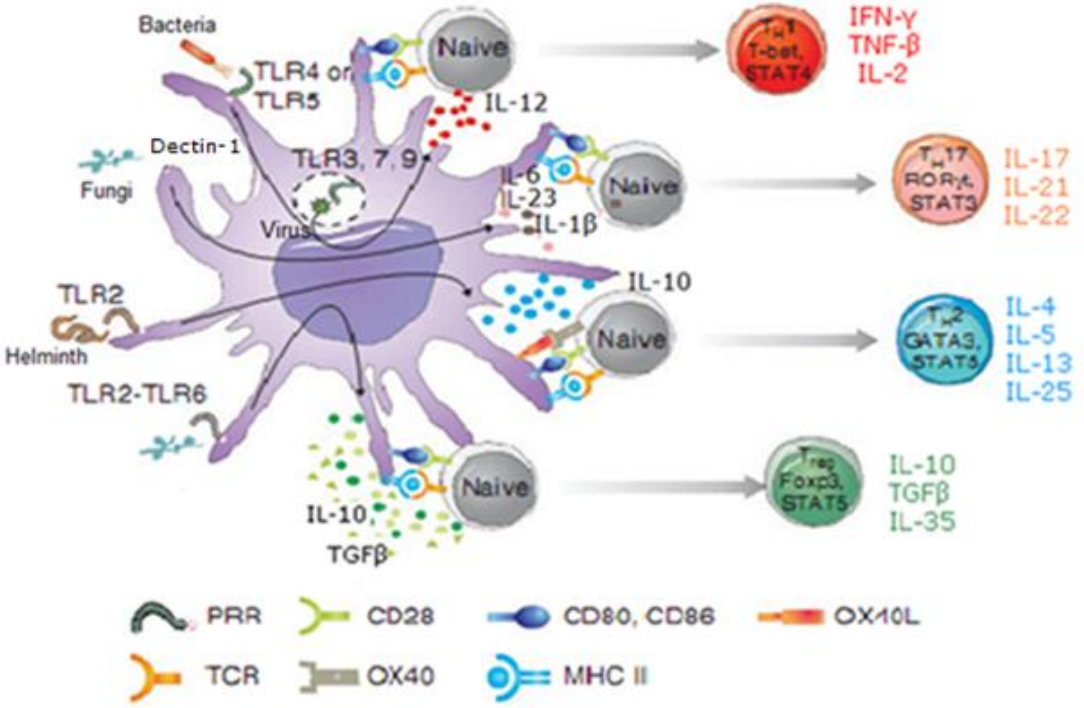

Figure 2. Interaction between dendritic cells and CD4$^+$ T cells.
The classical view of how DCs can stimulate the differentiation of naïve CD4$^+$ T cells into Th1, Th2, Th17 and the T regulatory cells and their cytokine production. TLR, toll-like receptor. Figure adapted from (Pulendran*, et al.*, 2010).

DCs produce large amounts of IL-12 as a result of their activation, e.g. through either a combination of TLR3, TLR4, TLR7, TLR8, TLR9, and TLR11 stimulation or a single TLR activation in the presence of type I IFNs, IFN-γ, or CD40L mediated signaling. They promote Th1 cell differentiation and proliferation (Zhu*, et al.*, 2008), with the CD40-CD40L interaction allowing the full capacity of mature DCs to produce IL-12 and to drive Th1 responses (De Jong *et al.*, 2005). IL-12 production contributes to tissue inflammation and induces or enhances IFN-γ production by Th1 and NK cells (De Jong*, et al.*, 2005). IFN-γ induces cell-mediated immunity against intracellular pathogens, directly inhibiting viral replication in infected cells and enhancing cytotoxic activity of macrophages (Kadowaki *et al.*, 2002; Tesmer *et al.*, 2008; Zhu*, et al.*, 2008). Th1 cells play a particularly important role in resistance to mycobacterial infections (Giacomini *et al.*, 2001). Th1 cells are also responsible for the induction of some autoimmune diseases (Zhu*, et al.*, 2008). Their principal cytokine products are IFN-γ, TNF-β, and IL-2 (figure 2) (De Jong*, et al.*, 2005; Zhu*, et al.*, 2008).

IL-4 is a specialized cytokine for the differentiation of Th2 cells. The main producers of IL-4 are believed to the mast cells and NKT cells. As DCs do not secrete IL-4, it is not clear whether or how they are involved in Th2 differentiation. It is considered that if their IL-12 secretion is suppressed it will initiate Th2 responses instead of Th1 responses (Lipscormb*, et al.*, 2002; O'Neill*, et al.*, 2004). Th2

cells mediate host defence against extracellular parasites including helminths. They are important in the induction and persistence of asthma and other allergic diseases. Th2 cells produce many cytokines, such as IL-4, IL-5, IL-13, and IL-25 (figure 2) (Jelley-Gibbs *et al.*, 2008; Miossec *et al.*, 2009; Zhu*, et al.*, 2008). IL-4 acts as a positive feedback cytokine for Th2 cell differentiation and a negative feedback cytokine for Th1 differentiation and is the major mediator of IgE class switching in B cells.

Recently, a new subtype of the effector $CD4^+$ T cell family, known as Th17, was identified. Differentiation of Th1 and Th2 cells follows similar rules in humans and mice, but Th17 differentiation may not be as conserved (Tesmer*, et al.*, 2008). It is clear that mice need IL-6 and TGFβ as factors for differentiation of Th17 cells with the cytokines IL-12, IFN-γ and IL-4 being able to inhibit Th17 differentiation in both mice and humans (Romagnani, 2008; Tesmer*, et al.*, 2008). More recently, IL-25 and IL-27 were also found to inhibit differentiation of Th17 cells (Romagnani, 2008). It has been revealed that the role of TGFβ in humans may not be central to Th17 differentiation but it is not clear yet which cytokines need to be secreted with IL-6 for the differentiation of human Th17 cells, most likely IL-1β and, some papers also mention IL-23 with IL-1β (figure 2) (Crome *et al.*, 2009; Miossec *et al.*, 2009; Romagnani, 2008; Tesmer*, et al.*, 2008; Zhu*, et al.*, 2008). Although TGFβ has not been considered to be central to Th17 differentiation in humans, three groups have recently reported that TGFβ may be critical for human Th17 cell differentiation (Zhu and Paul, 2008).

Th17 cells are pro-inflammatory cells characterized by the expression of IL-17a, IL-17f, IL-21, IL-22, and the transcription factor ROR-γt (figure 2) (Osorio *et al.*, 2008; Tesmer*, et al.*, 2008; Zhu*, et al.*, 2008). Both IL-17a and IL-17f recruit and activate neutrophils during immune response against extracellular bacteria and fungi, in which granulocyte infiltration is highly protective. IL-21 made by Th17 cells is a stimulatory factor for Th17 differentiation and serves as the positive feedback amplifier, as does IFN-γ for Th1 and IL-4 for Th2 cells. IL-21 also acts on $CD8^+$ T cells, B cells, NK cells, and DCs. Th17 cells are also responsible for, or participate in, the induction of many organ-specific autoimmune disease (Osorio*, et al.*, 2008; Romagnani, 2008; Zhu*, et al.*, 2008) but Th17 cells play a central role in autoimmune diseases, such as multiple sclerosis, rheumatoid arthritis, asthma, and inflammatory bowel disease (Tesmer*, et al.*, 2008). IL-22 mediates IL-23-induced acanthosis and dermal inflammation and IL-22 also protects hepatocytes during acute liver inflammation (Zhu*, et al.*, 2008).

Tregs play a major role in the maintenance of tolerance against self or harmless foreign proteins by down-regulating effector T cell responses that might erroneously have arisen to these proteins and thus prevent autoimmunity and unnecessary inflammation (De Jong*, et al.*, 2005; Osorio*, et al.*, 2008; Weaver*, et al.*, 2006; Zhu*, et al.*, 2008). Natural Tregs (nTregs) mature in the thymus and they are characterized by CD25 and Foxp3 expression (Murphy*, et al.*, 2008; Sakaguchi, 2005). Inducible Tregs (iTregs) are a group of T regulatory cells that differentiate in the periphery from naïve $CD4^+$ T cells (Murphy*, et al.*, 2008). Amongst the cytokines that induce iTreg differentiation are IL-10 and

TGFβ (Murphy*, et al.*, 2008; Stassen *et al.*, 2004). Amongst iTregs are the Th3 cells identified in the mucosal immune system that produce TGFβ, IL-10 and IL-4 and the Tr1 cells, which have mainly been characterized *in vitro*. Tr1 cells are induced by IL-10 and they secrete TGFβ (Murphy*, et al.*, 2008; Zhu*, et al.*, 2008). More recently another population of iTreg cells has been described in which Foxp3 expression is induced in naïve $CD4^+$ T cells in the presence of TGFβ and these iTreg cells secrete TGFβ, IL-10 and IL-35 (figure 2) (Murphy*, et al.*, 2008; Zhu*, et al.*, 2008). The relationship between the newly described iTreg cells and Th3 and Tr1 cells is currently unclear (Murphy*, et al.*, 2008). Through secretion of IL-10, TGFβ and IL-35, iTreg cells suppress other cells that mediate inflammation (Zhu*, et al.*, 2008).

Recently, other $CD4^+$ T effector cells have been described. These are the Th22 cells that are characterized by IL-22 secretion and the follicular helper T cells (Tfh cells) that have been shown to reside in the B cell follicles and participate in B cell activation (Sallusto *et al.*, 2009).

1.2.4 *In vitro* dendritic cell model

Characterization of DCs began about 40 years ago. During the first 20 years, they were painstakingly isolated from tissues and studied by few investigators. A low frequency of DCs in the blood made it difficult to isolate them in the quantity needed for research but the discovery of techniques to generate DCs *in vitro* allowed the identification of many of their biological and molecular properties (Uneo*, et al.*, 2007). Three types of cells have been used to culture DCs *in vitro*, myeloid progenitor cells ($CD34^+$ stem cells) isolated from bone marrow or umbilical-cord blood, blood plasmacytoid cells, or $CD14^+$ monocytes from blood (Shortman *et al.*, 2002). $CD14^+$ monocytes and $CD34^+$ stem cells have been most frequently used in *in vitro* studies of DCs but blood monocytes are most frequently used for generation of human DCs for immunotherapy. To generate DCs from $CD14^+$ monocytes, the monocytes are isolated from peripheral blood and incubated with granulocyte macrophage-colony-stimulating factor (GM-CSF) and IL-4 to let them differentiate into immature DCs with many characteristics similar to $CD11c^+$ DCs *in vivo*. Final maturation of the DCs is achieved by stimulating them with TNF-α and IL-1β, with or without extra stimulation such as LPS (Osugi *et al.*, 2002; Palucka, 1999; Shortman*, et al.*, 2002). CD14-derived DCs and CD34-derived DCs are morphologically, phenotypically and functionally similar but differences can be seen in the expression levels of some cell surface molecules, such as CD86 and HLA-DR. Both $CD14^+$ monocytes and $CD34^+$ stem cells are considered to be equally proficient to use for DCs studies *in vitro* (Syme *et al.*, 2005).

1.3 Natural products

Nature is rich of various products which have been used to ameliorate symptoms and cure human diseases since the dawn of medicine (Koehn *et al.*, 2005). Until about 200 years ago, when F. Setüner isolated morphine from opium, the majority of medicines available were crude drugs derived from plants (Cragg *et al.*, 2005; Samuelsson *et al.*, 2009). This discovery initiated a new era in the history of medicine. Natural products which are not pure compounds are in pharmacognosy termed as crude drugs. Crude drugs are unrefined medications in their raw or natural form, most of them are obtained from plants or parts of plants, extracts and exudates and some are of animal or insect origin (Samuelsson, *et al.*, 2009). Natural products have been the source of many of the active substances of Western medicine and many revolutionary drugs in use today are of natural origin (Harvey, 2000). Natural products are often divided into primary metabolites and secondary metabolites. Primary metabolites are mainly carbohydrates, proteins, fats and nucleic acids and are directly involved in normal growth, development, and reproduction (Dewick, 2009). Secondary metabolites are produced by organisms to cope with the challenge of their local environment and to gain advantages in the competition for living space and nutrients (Samuelsson, *et al.*, 2009).

Natural products are rich source of secondary metabolites and many of them have been shown to be pharmacologically active (Samuelsson, *et al.*, 2009). Although there has been a great expansion in synthetic chemistry, combinatorial chemistry and high throughput screening as well as in biotechnology in recent years, the success of using these methods to find new chemical entities have been limited. However, the importance of natural products as new drug leads has not decreased. More than one third of all pharmacologic agents in use today are of natural origin (Harvey, 2000; Harvey, 2008; Koehn, *et al.*, 2005; Li *et al.*, 2009) and thereof about 60 to 70% of antibacterial and anticancer drugs are derived from nature (Lam, 2007; Cragg *et al.*, 2009).

1.3.1 Traditional medicine

In the past ancient civilizations depended greatly on local flora and fauna for their survival. As a result, many crude drugs were observed to have some medical use (Barnes *et al.*, 2007). In some Asian and African countries today, 80% of the population relies on traditional medicine for primary health care (Who, 2008). In developing countries, the use of traditional medicine for treatment of various types of ailments, to improve their state of health and for preventive measures is considerable and has increased steadily in recent years (Samy *et al.*, 2010) especially among individuals with chronic inflammatory diseases and cancer (Barnes, *et al.*, 2007; Fetrow *et al.*, 2004). New drugs can be discovered as a result of research programmes aimed at the study of medicinal plants used in traditional medicine; this drug research has been termed ethnopharmacology (Samuelsson, *et al.*, 2009). Ethnopharmacology is the scientific study correlating ethnic groups, their health and how it relates to their physical habits and methodology in creating and using medicines (Johnson *et al.*, 1996). An example where this approach has been successful is the antimalarial drug artemisinin,

which was isolated from a plant (*Artemisia annua L.)* that for at least 1700 years has been used in traditional Chinese medicine for treatment of fever, including fever caused by malaria (Samuelsson*, et al.*, 2009; Wang *et al.*, 2007). Water or ethanol are the main solvents used for crude extract preparations. Crude extracts or mixtures of compounds are used for the initial screening of plants (Samy*, et al.*, 2010).

1.3.2 Natural products and immunomodulating activity

Traditional medicine is most commonly used in inflammatory diseases. However, for many of the plants in use, the real efficacy and/or the relevant active components are unknown (Erdemoglu *et al.*, 2003). The anti-inflammatory drugs presently available for the treatment of various inflammatory disorders have one or more adverse or undesirable side-effects (Geetha *et al.*, 2001) and consequently discovery of new anti-inflammatory drugs with less side effects would be beneficial. Plants with anti-inflammatory effects might serve as leads for the development of such drugs (Geetha *et al.*, 2001). Plant-derived anti-inflammatory compounds could even be used in combination with pharmaceuticals in order to lower the concentration used and subsequently the adverse effects.

Some of the plants used in traditional medicine have been shown to possess various biological effects, including immunomodulatory activity (Kim *et al.*, 2007). The majority of studies describing immunomodulating activity of natural products have focused on their effects on the innate arm of the immune system but only a few studies have been performed to determine the effects of natural products on DCs. Recently, extract of seeds of *Plantago asiatica* L. was shown to be able to induce maturation of mouse DCs, providing a theoretical support for the use of this plant in traditional Chinese medicine for patients suffering from chronic illnesses (Huang *et al.*, 2009). Furthermore, polysaccharides from the mushroom *Ganoderma lucidum* had various effects on human monocyte-derived DCs depending on whether they were derived from mycelium or spore (Chan *et al.*, 2007).

In Iceland, several studies on bioactivity of natural products have been performed and some of them have shown the natural product to have immunomodulating activity (Freysdottir *et al.*, 2008; Olafsdottir *et al.*, 2003; Omarsdottir *et al.*, 2006). Several studies have also been conducted using a human *in vitro* DC model to screen natural products for their immunomodulating effects. In these studies, polysaccharides from lichens, alkaloids from club moss, and extracts from marine invertebrates as well as a number of other extracts, fractions and pure compounds showed interesting immunomodulating activity. In a study where eleven polysaccharides of different structural types were isolated from Icelandic lichens and their effects on DCs maturation *in vitro* was analysed, four lichen polysaccharides had the potential to induce anti-inflammatory effects (Omarsdottir*, et al.*, 2006). In another study, the aqueous extract of the Icelandic lichen *Cetraria islandica* had anti-inflammatory effect in an arthritis model in rats (Freysdottir*, et al.*, 2008). Furthermore, ethanol extract from birch bark was shown to contain components that can affect the stimulation of DCs to a less active phenotype resulting in reduced Th1 type response (Sigurpalsson, unpublished work). Marine natural

products chemistry is comparatively new compared to phytochemistry and the terrestrial biota has been investigated to some extent while large areas of the oceans are still unexplored. In a study were effects of the omega-3 fatty acid eicosapentaenoic acid (EPA) on the maturation of DCs *in vitro* was investigated, treatment of DCs with EPA resulted in fewer DCs with mature phenotype, although the DCs seemed to be fully capable of activating and differentiating T cells (Stefansdottir, unpublished work). In addition, results from a recent study indicated that extracts of Icelandic marine invertebrates contain bioactive substances (Finnsson, unpublished work).

1.3.3 Polysaccharides

Polysaccharides are carbohydrates, containing repeating mono- or disaccharide units. Polysaccharides can be homoglycans or heteroglycans. Homoglycans are polymers containing one type of monosaccharide residue, whereas heteroglycans are polymers of more than one type of monosaccharide residue. Polysaccharides are often quite heterogeneous, containing slight modifications of the repeating unit. These polymers can either have linear or branching structure (Paulsen *et al.*, 2005). Polysaccharides are important as structural material for plant cells and for crustaceous animals and are, probably, the most abundant, naturally occurring component. The cell walls of all plants are composed of polysaccharides and the majority of these fall into the group called pectin (Paulsen, 2002; The Review of Natural Products, 2009).

Pectin polymers are roughly divided into arabinogalactans, rhamnogalacturonans and comaruman and they have a variable molecular weight ranging from 20.000-400.000. Pectin polymers have been evaluated for their effects on the immune system as have other polysaccharides obtained from various plants, such as *Plantago major* L (Samuelsen *et al.*, 1996) and *Glinus oppositifolius* (Inngjerdingen et al., 2007). A recent PhD study showed that pectic polymers isolated from three Malian medicinal plants, *Biophytum petersianum*, *Combretum glutinosum* and *Opilia celtidifolia,* that have been used in treatment of various ailments had immunomodulating activity that may, at least partly, be responsible for the medicinal effects of these plants (Grönhaug, 2010).

Many of the herbal remedies used in traditional medicine contain bioactive polysaccharides (Paulsen, *et al.*, 2005). Polysaccharides have for a long time been thought to be important when using herbs in traditional medicine in different parts of the world for treating various diseases (Paulsen, 2002). Usually, the medicinal plants are consumed as tea, infusions or decocations, prepared by boiling the plants in water. These preparations contain a lot of polysaccharides which probably have some bioactivity, and are not just building stones in the cell wall (Paulsen, 2002; Paulsen, *et al.*, 2005).

1.3.4 Geranium, *Geranium sylvaticum,*

Geranium *(Geranium sylvaticum),* belonging to the family Geraniaceae, is also known as wood cranesbill or woodland geranium and in Iceland as blágresi (figure 3). Geranium is widespread in Iceland, as well as in Europe and North America. The plant grows in the undergrowth of forests, on hillsides shielded from winds, in gullies and ravines (Kristinsson, 2010; Fetrow*, et al.*, 2004). When the plant is used in folk medicine it is collected before blossom and the whole plant with the root is used (Johannsdottir, 2009; Kristinsson, 2010). The book by Johannsdottir is used as a reference for indications of use of plants in traditional medicine in Iceland, although it is not a scientific reference.

Figure 3. Geranium, *Geranium sylvaticum.*
(Kristinsson, 2010).

No phytochemical analysis was found for *Geranium sylvaticum* and it is seldom referred to as a medicinal herb in recent literature. Very few studies have focused on the bioactivity of the plant but an Icelandic study has demonstrated that ethanol extracts of *Angelica archangelica* seeds and the aerial parts of *Geranium sylvaticum* synergistically inhibited the activity of acetylcholinesterase (Sigurdsson *et al.*, 2007).

Geranium sylvaticum has been used traditionally for centuries and has been considered beneficial against inflammation, gastrointestinal sores, and all kinds of arthritis, especially gout (Johannsdottir, 2009). *Geranium maculatum* from the same family has also been used to ameliorate similar ailments *(Database, 2010; Fetrow, et al., 2004).*

1.3.5 Bogbean, *Menyantes trifoliate*

Bogbean (*Menyanthes trifoliata),* belonging to the family Menyanthaceae, is known in Iceland as horblaðka (figure 4). Bogbean is widespread all around Iceland and throughout Europe and North America. It grows in mire, ditches and shallow ponds (Kristinsson, 2010; Fetrow*, et al.*, 2004). The plant is collected early in the summer and the leaves are used in folk medicine (Barnes*, et al.*, 2007; Database, 2010; Fetrow*, et al.*, 2004; Johannsdottir, 2009). The main chemical constituents of bogbean are shown in table 1.

Figure 4. Bogbean, *Menyanthes trifoliate.*

Bogbean has been used traditionally through the ages to cure all kinds of inflammations, e.g. rheumatoid arthritis and it was used in the past against scurvy. The plant is considered to have stimulating effect upon the colon, acting as an aperient and should not be used by individuals with diarrhoea, dysentery or colitis (Database, 2010; Fetrow*, et al.*, 2004; Johannsdottir, 2009). Bogbean is listed by the Council of Europe as a natural source of food flavouring (category N2) (Barnes*, et al.*, 2007).

Table 1. The chemical constituents of bogbean.

Acid	Alkaloids	Coumarins	Flavonoids	Iridoids	Other constituents
Caffeic -, chlorogenic -, ferulic -, *p.*hydroxyben - zoic -, protocatechuic - ,salicylic-, vanillic -, folic- and palmitic acid.	Gentianin, and gentianidine.	Scopoletin.	Hyperin, kaempferol, quercetin, rutin and trifolioside.	7′,8′-Dihydro-folamenthin, foliamenthin, loganin, menthiafolin and sweroside	Carotene, ceryl-alcohol, enzymes and α-spinasterol

(Barnes*, et al.*, 2007)

1.3.6 Meadowsweet, *Filipendula ulmaria*

Meadowsweet (*Filipendula ulmaria*) is in the family Rosaceae and is known in Iceland as mjaðjurt (figure 3). Meadowsweet is native to South- and Southwest Iceland but is less common in other regions. It also grows in Europe, Northern Asia and North America. It grows in damp meadows, bogs and in damp open woodland (Kristinsson, 2010; Fetrow, *et al.,* 2004). The plant is collected during midsummer and the leaves and flower are used (Database, 2010; Fetrow*, et al.*, 2004; Johannsdottir, 2009). A list of identified constituents of meadowsweet is shown in table 2.

Figure 5. Meadowsweet, *Filipendula ulmaria*.

Meadowsweet has been used traditionally for ages and is considered to ameliorate gastritis, arthritis, fibrositis and neuritis and is also used as a supportive treatment for common cold. The plant has both been called a friend of the stomach and the herbalist's painkiller (Database, 2010; Fetrow*, et al.*, 2004; Johannsdottir, 2009; The Review of Natural Products, 2005). Previously, meadowsweet has been listed by the Food and Drugs Administration (FDA) as a Herb of Undefined Safety, as there is a lack of clinical research assessing the adverse effects of meadowsweet (Barnes*, et al.*, 2007).

Table 2. The chemical constituents of meadowsweet.

Flavonoids	Salicylates	Tannins	Volatile oils*	Other constituents
Flavonols, flavones, flavanones and chalcone derivatives.	Salicylaldehyde,* gaultherin, isosalicin, methyl salicylate, monotropitin, salicin, salicylic acid and spirein.	1% (alcoholic-extract), 12.5% (aqueous extract) and catechols (hydrolysable type).	Benzyl alcohol, benzaldehyde, ethyl benzoate, heliotropin, phenylacetate and vanillin*.	Coumarin, mucilage, carbohydrates and ascorbic acid

* Main components of the volatile oil including salicylaldehyde (major, up to 70%) (Barnes*, et al.*, 2007).

1.3.7 Yarrow, *Achillea millefolium*

Yarrow (*Achillea millefolium*) is in the family Asteraceae and is known in Iceland as vallhumall (figure 4). Yarrow is native and widespread throughout Iceland, most of Europe, North America and Asia. The plant grows in dry banks and fields and is often found around roads and towns (Kristinsson, 2010; Fetrow*, et al.,* 2004). The plant is collect in midsummer and the leaves and flowers are used (Database, 2010; Fetrow*, et al.*, 2004; Johannsdottir, 2009). The main chemical constituents of yarrow are shown in table 3.

Figure 6. Yarrow, *Achillea millefolium.*

Yarrow has been used traditionally for centuries and has been considered good for healing wounds, diaphoresis, high blood pressure and last, but not least, rheumatoid arthritis. Common trade name for yarrow is rheumatic pain remedy (Database, 2010; Fetrow*, et al.*, 2004; Johannsdottir, 2009; The Review of Natural Products, 2004). Yarrow is listed by the Council of Europe as a natural source of food flavouring (Barnes*, et al.*, 2007). It has, however, never been tested in clinical studies for effects on diseases and should be used cautiously in view of its apparent tendency to cause contact dermatitis (Fetrow*, et al.*, 2004).

Table 3. The chemical constituents of yarrow.

Acids	Alkalids/bases	Flavonoids	Tannins	Volatile oils	Other constituents
Amino acids and fatty acids.	Betonicine and stachydrine, trigonelline, betaine and choline.	Flavone glycosides apigenin and luteolin-7-glycosides.	Condensed and hydro-lysable with glucose as the carbo-hydrate component of the latter.	Borneol, bornyl-acetate, camphor, 1,8-cineole, eucalyptol, limonene, sabinene, terpinen-4-ol, terpineol and α-thujone	Arabinose, galactose, dextrose, dulcitol, glucose, inositol, maltose, mannitol and sucrose.

(Barnes*, et al.*, 2007).

2 Aim

Bogbean, geranium, meadowsweet and yarrow, have been used in traditional medicine for centuries to ameliorate various diseases, such as rheumatoid arthritis. The aim of this study was to investigate whether extracts of these plants have anti-inflammatory effects.

Specific aims were:

1. To determine the effects of ethanol extracts of the plants on:
   a. Maturation of human DCs *in vitro.*
   b. The ability of DCs matured in presence of the extracts to activate allogeneic $CD4^+$ T cells.

2. To determine the effects of aqueous extracts of the plants on:
   a. Maturation of human DCs *in vitro*.
   b. The ability of DCs matured in presence of the extracts to activate allogeneic $CD4^+$ T cells.

3 Material and methods

3.1 Plant extracts

3.1.1 Plants

The four plants used in this project were collected during the summer of 2008. Geranium was collected in Heiðmörk in late June. Bogbean and meadowsweet were collected in Mýrarkotsland, Grímnes, bogbean in the middle of June and meadowsweet in the middle of July. Yarrow was collected at Hvanneyri in early July. Voucher specimens were stored at The Icelandic Institute of Natural History, Akureyri (appendix 1, document 1). The plants were allowed to dry at room temperature (RT) and stored in a dry, dark place until used.

3.1.2 Preparation of extracts

Two types of extracts were prepared from the dried plant material. Ethanol extracts were prepared by shaking 5 g of the plant material in 100 mL of ethanol on a plate shaker for 24 hours at RT, followed by filtration and evaporation of the solvent. Before use, the extracts were redissolved in 10% dimethyl sulfoxide (DMSO) and 90% RPMI 1640 medium (Invitrogen, UK) containing 10% foetal calf serum (Invitrogen) and penicillin/streptomycin (Invitrogen) (culture medium). Water extracts were prepared by shaking 100 g of the plant material in 1.5 L of 85°C distilled water for two hours, followed by filtration and freeze-drying. The extracts were redissolved in culture medium and allowed to tilt on a lab roller for one hour at RT before use.

3.2 Preparation of DCs

3.2.1 Isolation of peripheral blood mononuclear cells

Blood was collected from healthy volunteers (ethical approval number 06-068) into EDTA-containing tubes. Peripheral blood mononuclear cells (PBMCs) were isolated by adding the blood sample to a Ficoll-Paque PLUS medium which separates cells according to density and centrifuging at 1600 rpm for 30 minutes at 22ºC without using brakes. The PBMCs were washed three times with magnetic activated cell sorting (MACS) buffer (PBS containing 0.5% bovine serum albumin (BSA) (Millipore, USA) and 2 mM ethylenediaminetetraacetic acid (EDTA)) and centrifuged at 1300 rpm for 10 minutes at 4ºC. Before the third wash, the cells were counted in 0.4% trypan blue (1:6) using improved Neubauer chamber.

3.2.2 Isolation of $CD14^+$ monocytes

The PBMCs were diluted in MACS buffer to a final concentration of 12.5 x 10^7 cells/mL. The monocytes were isolated using CD14 MicroBeads (Miltenyi Biotec, Germany), where microbead-conjugated antibodies against CD14, which is present on monocytes, were added to the cells and the solution left on ice for 15 minutes. The cells were then washed with 20 mL MACS buffer and centrifuged at 1300 rpm for 10 minutes at 4ºC. The cells were diluted in MACS buffer, 500 µL, for each 1 x 10^8 cells. The cells were passed through a column (Miltenyi Biotec), placed in a strong magnetic field, in which the microbead-labelled cells were retained. The column was washed three times with MACS buffer in order to remove all unlabelled cells. Finally the column was removed from the magnetic field and the $CD14^+$ cells collected from the column. Following counting in 0.4% trypan blue (1:6) using improved Neubauer chamber, the cells were centrifuged at 1300 rpm for 10 minutes at 4ºC.

3.2.3 Differentiation of $CD14^+$ monocytes to immature DCs

The cells were diluted in culture medium to a concentration of 0.5 x 10^6 cells/mL. An aliquot of the cells was removed for determination of CD14 expression by flow cytometry (see later). The cells were plated on 48-well tissue culture plates, 1 mL/well, and IL-4 at 12.5 ng/mL (R&D Systems, England) and GM-CSF at 25 ng/mL (R&D Systems) added to stimulate monocyte differentiation into imDCs. The cells were incubated at 37ºC, in 5% CO_2 and 100% humidity, for 7 days. On day 3 or 4, 0.5 mL of culture medium, containing the same quantity of IL-4 and GM-CSF as before, was added to the cells (figure 7).

3.3 Maturation of DCs

At day 7 the imDCs were examined in a microscope and harvested. Following counting in 0.4% trypan blue (1:1) using improved Neubauer chamber, the cells were centrifuged at 1300 rpm for 10 minutes at 4ºC. An aliquot of the supernatant was collected and stored at -80ºC for analysis of cytokines by enzyme-linked immunosorbent assay (ELISA) (see later). The imDCs were diluted in culture medium to a final concentration of 2.5 x 10^5 cells/mL. An aliquot of the cells was removed and stained with fluorescence-labelled antibodies and surface expression of DC markers analysed by flow cytometry (see later).

The imDCs were added to 48-well tissue culture plates, 500 µL/well and IL-1β at 10 ng/mL (R&D Systems) and TNF-α at 50 ng/mL (R&D Systems) added to all wells. All imDCs were activated with lipopolysaccharide (LPS) at 500 ng/mL (Sigma, Germany). Vitamin D3 (Biomol International, USA) at 4 x 10^{-8} M, was added to the cells as a control because vitamin D3 is known to induce DCs with tolerogenic properties, e.g. with reduced IL-12 secretion (Adorini *et al.*, 2004). The plant extracts were added to the cells at final concentrations of 0.1, 1, 10, 25, 50 and 100 µg/mL. As DMSO was used to

solubilise ethanol extracts from the plants in the culture medium, the highest concentration of DMSO used was also added to the cells as a control. The imDCs were incubated at 37ºC, in 5% CO_2 and 100% humidity, for 2 days. On day 9, the imDCs had matured into DCs and were examined in a microscope and harvested. The cells were centrifuged at 1300 rpm for 10 minutes at 4ºC and the supernatants collected and stored at -80ºC for determination of cytokine concentration by ELISA (see later). The DCs were stained with fluorescence-labelled antibodies and surface expression of DC markers analysed by flow cytometry (see later).

3.4 Co-culture of DCs and allogeneic $CD4^+$ T cells

The effects of maturing DCs in the presence of plant extracts from bogbean and yarrow on the ability of the DCs to stimulate allogeneic $CD4^+$ T cells, was analysed. The experimental set-up is shown in figure 7.

3.4.1 Isolation of allogeneic $CD4^+$ T cells

Allogeneic $CD4^+$ T cells were isolated from peripheral blood in the same manner as described for monocytes (see sections 7.2.1 and 7.2.2) except using CD4 MicroBeads instead of CD14 MicroBeads. Following isolation of $CD4^+$ T cells, the cells were dissolved in culture medium at a concentration of 2×10^6 cells/mL. An aliquot of the cells was removed for determination of CD4 expression by flow cytometry (see later).

3.4.2 Maturation of DCs

ImDCs were matured in the same manner as before, with IL-1β, TNF-α and LPS (figure 7). Plant extracts from yarrow and bogbean were added to the cells at a concentration of 50 µg/mL. On day 9, the DCs were examined in a microscope, harvested and counted as before. DCs were diluted in culture medium at a concentration of 2×10^5 cells/mL.

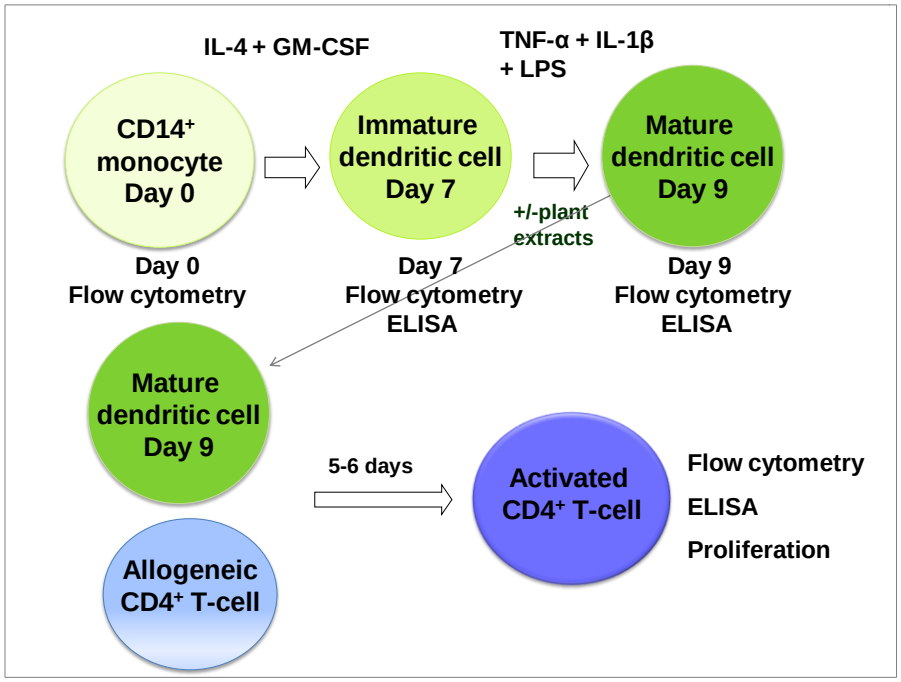

Figure 7. Schematic presentation of the experimental set-up.

3.4.3 Co-culture of DCs and allogeneic $CD4^+$ T cells

DCs matured in the presence of extracts from bogbean (B-DCs) or yarrow (Y-DCs) or without extracts (mDCs) were cultured with allogeneic $CD4^+$ T cells in 96-well round bottom culture plates. The number of $CD4^+$ T cells was kept constant at 2 x 10^5 cells/well, whereas the number of DCs was 2 x 10^4 cells/well (DC:T cell ratio of 1:10) for cytokine measurements and flow cytometry but 0.33 x 10^4 cells/well (DC:T cell ratio of 1:67), 0.11 x 10^4 cells/well (DC:T cell ratio of 1:200), 0.037 x 10^4 cells/well (DC:T cell ratio of 1:600) and 0.012 x 10^4 cells/well (DC:T cell ratio of 1:1800) for determination of T cell proliferation. For comparison, T cells at 2 x 10^5 cells/well and DCs at 2 x 10^4 cells/well were cultured alone. The plates were incubated at 37ºC, 5% CO_2 and 100% humidity for 6 days. For cytokine secretion and flow cytometric analysis, the cells were examined in a microscope, harvested and centrifuged. The supernatants were collected and stored at -80°C for later use. The cells were stained with fluorescence-labelled antibodies and analysed by flow cytometry (figure 7).

3.4.4 Proliferation of T cells

To determine proliferation of the T cells, 0.5 µCi ^{3}H-thymidine (Amersham, GE Healthcare, England) was added to each well on day 4 and the cells cultured for further 16 hours. Cells were then harvested on filter paper (Omnifilter, Packard, CA) using a cell harvester (Filtermate, Packard). The filter paper was placed in a counting plate and 30 µL MicroScint-O cocktail (Packard) added to each well. The radioactivity bound to the filters was determined using a scintillation counter (TopCount, Packard), which presents data as mean counts per minute (cpm).

3.5 Expression of surface molecules and secretion of cytokines

Cell samples were taken after purification of $CD14^+$ monocytes, differentiation of imDC, maturation of DCs, purification of allogeneic $CD4^+$ T and co-culturing of allogeneic $CD4^+$ T cells with DCs and the expression of surface molecules analysed by flow cytometry and cytokine concentration in the supernatants determined by ELISA (figure 7).

3.5.1 Determination of expression of surface molecules on monocytes, DCs and allogeneic $CD4^+$ T-cells

The surface molecules that were examined on monocytes and DCs were CD14, CD86, HLA-DR, CCR7 and DC-SIGN and for allogeneic $CD4^+$ T cells the molecules CD4, CD25 and CD54 (ICAM-1). The cells were divided into tubes, approximately 100 µL of 0.5 x 10^5 cells in each tube and 5 µL of fluorescence-labelled monoclonal antibodies, diluted according to instructions from the manufacturers, added to the tubes (see tables 4 and 5). For control, cells were stained with isotype-specific mouse antibodies (tables 4 and 5).

Table 4. Fluorescence-labelled antibodies for determination of expression of surface molecules on monocytes and DCs.

Tube 1	Tube 2	Tube 3
IgG1 FITC[a]	CD86 FITC[a]	DC-SIGN FITC[a]
IgG2a PE[a]	HLA-DR PE[b]	CCR7 PE[c]
IgG2a APC[b]	CD14 APC[a]	

Table 5. Fluorescence-labelled antibodies for determination of expression of surface molecules on $CD4^+$ T cells and DCs in co-cultures.

Tube 1	Tube 2	Tube 3
IgG1 FITC[a]	CD25 FITC[b]	CD86 FITC[a]
IgG2a PE[a]	CD4 PE[b]	HLA-DR PE[b]
IgG2a APC[b]	ICAM-1 APC[b]	

[a] AbD Serotec (England); [b] BD Bioscience (USA), [c] R&D Systems. FITC, fluorescein isothiocyanate; PE, phycoerythrin; APC, allophycocyanin.

The cells were incubated with the antibodies for 20 minutes on ice, washed with 2 mL staining buffer (PBS with 0.5% BSA, 2 mM EDTA, 0.1% NaN_3) and centrifuged at 1200 rpm for 5 minutes at 4ºC. The cell pellet was resuspended and fixed in 300 µL of 1% paraformaldehyde in PBS and stored at 4ºC in the dark until analysed. Ten thousand cells were collected using FACScaliber (BD Biosciences, USA) and analysis performed using the CellQuest programme (BD Biosciences). The results are expressed either as percentage positive cells as compared with cells stained with isotype control antibodies or as mean fluorescence intensity (MFI).

Determination of CD14 expression confirmed 95% purity of the monocytes and reduction in CD14 expression and an increase in CD86 and HLA-DR expression confirmed differentiation of the monocytes into imDCs. Neither vitamin D3 nor DMSO, both used as controls, affected expression of surface molecules on DCs. Determination of CD4 expression confirmed 95% purity of $CD4^+$ T cells and was used to distinguish T cells from the DCs when staining the cells following co-cultures.

3.5.2 Determination of cytokine concentration in supernatants

Secreted cytokines were measured in the collected supernatants (see above) by sandwich ELISA. The cytokines IL-6, IL-10, IL-12p40 and IL-23 were measured in supernatants from DCs and IL-4, IL-6, IL-10, IL-12p40, IL-17, IL-23 and IFN-γ in supernatants from co-cultures of DCs and allogeneic $CD4^+$ T cells. For all cytokines except IL-23, DuoSet ELISA kits were used according to the manufacturer's protocol (R&D Systems). ELISA Maxisorp plates (Nunc, Denmark) were coated with purified mouse anti-human cytokine antibodies overnight at RT. The following day, unbound binding sites on the plates were blocked with PBS containing 1% BSA, 5% sucrose (Sigma) and 0.05% NaN_3 for 1 hour at RT. After blocking, the plates were washed with wash buffer (PBS containing 0.05% Tween 20 (Sigma)) and supernatants and standards added. The plates were incubated for 2 hours at RT and then washed. Biotinylated goat anti-human cytokine antibodies were added and the plates incubated for 2 hours at RT and then washed again. Then the plates were incubated with horseradish peroxidase (HRP)-conjugated streptavidin for 20 minutes at RT and following a final wash, the TMB ONE substrate solution (Kementec, Denmark) was added. The reaction was stopped by addition of 0.18 M sulphuric acid to each well and the absorbance measured at 450 nm in a microplate reader (MultiskanEX/Thermo, Finland). IL-23 kit was obtained from eBioscience (USA). The IL-23 ELISA was performed in the same manner as for the other cytokines, except that unbound sites on the plates were blocked with assay diluent for 1 hour at RT, incubation of supernatants and IL-23 standard was overnight at 4°C, incubation with biotinylated anti-human IL-23 antibody was for 1 hour and with HRP-conjugated streptavidin for 30 minutes.

Each sample was run in duplicate and assay diluent used as a blank. Values were calculated from a seven point standard curve constructed by serial 2-fold dilutions of respective recombinant human cytokines. The results are expressed as pg/mL or secretion index (SI), where cytokine secretion by DCs matured in the presence of extracts from plant was divided by cytokine secretion by DCs matured without extract. This was done to eliminate differences between individuals from which the blood was collected. To assess whether differences in cytokine secretion were pro- or anti-inflammatory, the ratio of SI for IL-12p40 and IL-10 was calculated. If there was an increase in IL-12p40 and/or a decrease in IL-10 secretion the ratio was higher than 1, whereas if there was a decrease in IL-12p40 and/or an increase in IL-10 secretion the ratio was lower than 1.

Maturing DCs in the presence of DMSO did not affect cytokine production, whereas vitamin D caused a reduction in IL-12p40 and an increase in IL-6 secretion by DCs.

3.6 Statistics

The mean, standard deviation and standard error was calculated for each treatment group and the difference between the groups compared by analysis of variance (ANOVA) using SigmaTest. A p-value of <0.05 was considered statistically significant.

4 Results

4.1 The effects of ethanol extracts from bogbean, geranium, meadowsweet and yarrow on maturation of DCs

4.1.1 Yield, appearance and texture of ethanol extracts from bogbean, geranium, meadowsweet and yarrow

Yield, appearance and texture of ethanol extracts from bogbean, geranium, meadowsweet and yarrow is shown in table 6.

Table 6. Quantity, yield, appearance and texture of ethanol extracts from bogbean, geranium, meadowsweet and yarrow.

Plants	Quantity Dry material	Yield Dry extract	Appearance and texture
Bogbean (leaves)	6.94 g	2.6% (183 mg)	Slightly dry and clumped
Geranium (leaves, flowers and root)	6.53 g	7.8% (507 mg)	Rather viscous and sticky
Meadowsweet (leaves and flowers)	6.04 g	5.7% (344 mg)	Slightly clumped and sticky
Yarrow (leaves and flowers)	6.04 g	3.0% (180 mg)	Rather dry and clumped

4.1.2 The effects of ethanol extracts from bogbean, geranium, meadowsweet and yarrow on DC viability

Ethanol extract from geranium did not affect viability of the DCs when used at a concentration of 1 and 10 µg/mL, but 50 and 100 µg/mL of ethanol extract from geranium decreased cell viability (table 7). Ethanol extracts from bogbean, meadowsweet and yarrow had similar effects (data not shown). Microscopic examination also showed that DCs matured in the presence of 50 and 100 µg/mL of the plant extracts were dying or dead (data not shown). DMSO, in the same concentrations as the extracts were dissolved in, did not affect cell viability (data not shown).

Table 7. The effect of extract from geranium on DC viability.

Concentration	Count (cells/ml)
100 µg/mL	4.1×10^4
50 µg/mL	2×10^4
10 µg/mL	96×10^4
1 µg/mL	126×10^4
0 (LPS only)	74×10^4

DCs were matured with or without different concentrations of ethanol extract from geranium and stimulated with IL-1β, TNF-α and LPS for 48 hours. Viable cells were counted using improved Neubauer counting chamber.

4.1.3 The effects of ethanol extracts from bogbean, geranium, meadowsweet and yarrow on expression of surface molecules on DCs

The effects of ethanol extracts from bogbean, geranium, meadowsweet and yarrow (0.1 to 25 µg/mL) on expression of CD14, CD86, CCR7, DC-SIGN and HLA-DR on DCs were examined.

Ethanol extracts from bogbean, geranium, meadowsweet and yarrow did not affect expression of CD14 on DCs (data not shown). The expression (proportion of cells expressing the molecule or MFI) of CD86 increased with differentiation of monocytes into imDCs and further maturation of imDCs into mDCs (figure 8). The expression of CCR7, DC-SIGN and HLA-DR also increased with differentiation of monocytes into imDCs and further with maturation of imDCs into DCs (data now shown). Ethanol extracts from geranium (figure 8), meadowsweet, bogbean and yarrow did not affect expression of CD86 on mDCs or expression of CCR7, DC-SIGN and HLA-DR (data not shown).

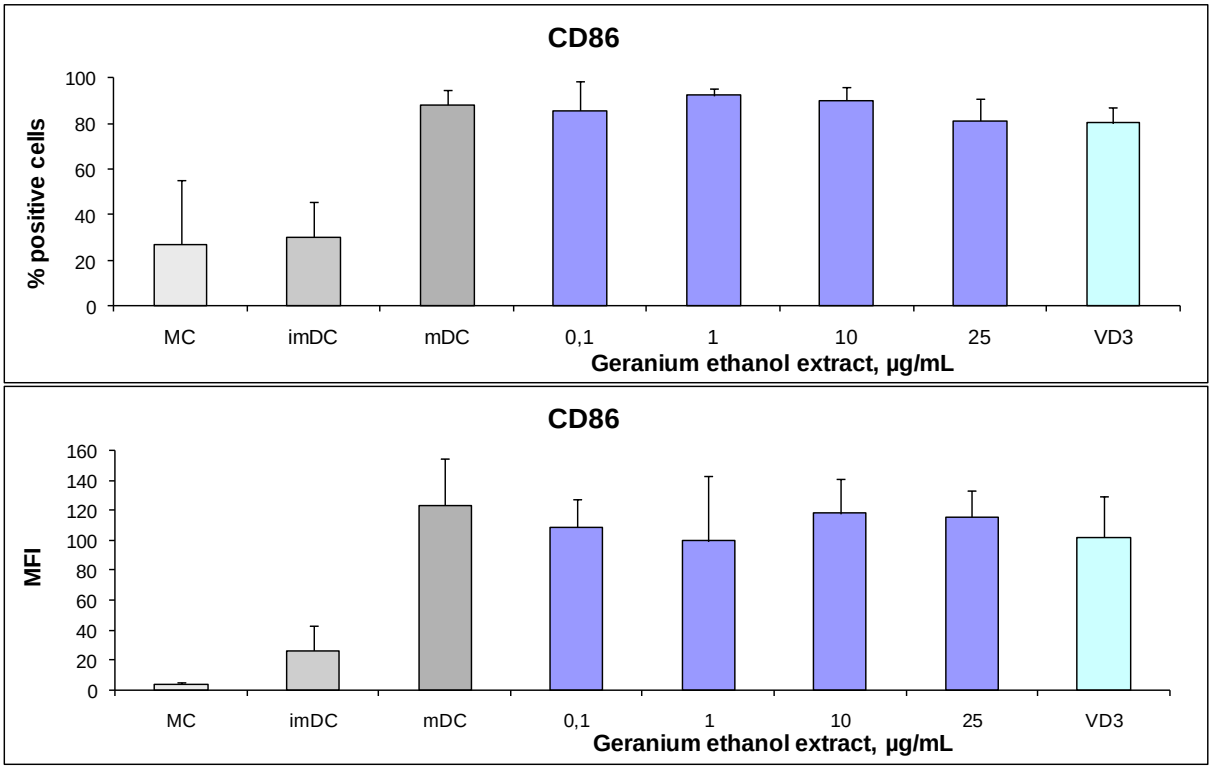

Figure 8. The effects of ethanol extracts from geranium on the proportion of cells expressing CD86 and mean fluorescence intensity of CD86 expression by DCs.
DCs were matured without or in the presence of ethanol extract from geranium at concentrations from 0.1 to 25 µg/mL. Results are shown as mean percentage of positive cells or MFI ± SEM, n=3-6. MC, monocytes; imDC, immature dendritic cells; mDC, mature dendritic cells; VD3, vitamin D3.

4.1.4 The effects of ethanol extracts from bogbean, geranium, meadowsweet and yarrow on cytokine secretion by DCs

Ethanol extract from geranium at concentrations from 0.1 to 25 µg/mL did not affect DC secretion of IL-12p40 (figure 9) nor did it affect DC secretion of IL-10 or IL-6 (data not shown). Ethanol extracts from bogbean, meadowsweet and yarrow did not affect secretion of any of the three cytokines (data not shown).

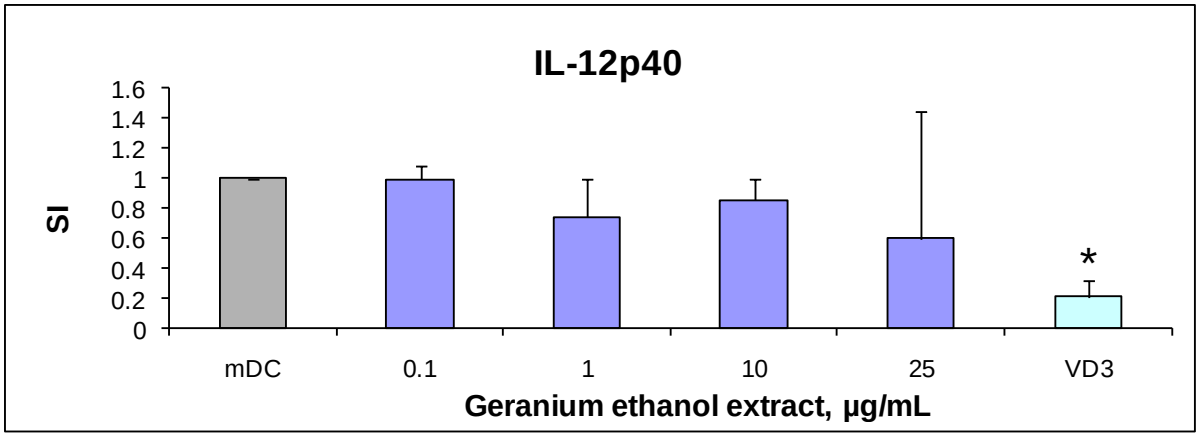

Figure 9. The effects of ethanol extracts from geranium on IL12p40 secretion by DCs.
DCs were matured without or in the presence of ethanol extract from geranium at concentrations from 0.1 to 25 µg/mL. Results are shown as SI ± SEM, n=4. mDC, mature dendritic cells; VD3, vitamin D3. * Different from DCs matured without plant extracts (mDC), $p<0.05$.

4.2 The effects of aqueous extracts from bogbean, geranium, meadowsweet and yarrow on maturation of DCs

4.2.1 Yield, colour and texture of aqueous extracts from bogbean, geranium, meadowsweet and yarrow

Yield, colour and texture of aqueous extracts from bogbean, geranium, meadowsweet and yarrow is shown in table 8.

Table 8. Quantity, yield, colour and texture of aqueous extracts from bogbean, geranium, meadowsweet and yarrow.

Plants	Quantity Dry material	Yield Dry extract	Colour and texture
Bogbean (leaves)	98 g	7.3% (7.14 g)	Light moss green foam
Geranium (leaves, stalks, flowers and roots)	104 g	13.9% (14.5 g)	Green yellow foam
Meadowsweet (leaves and flowers)	97 g	6.3% (6.13 g)	Yellow green foam
Yarrow (leaves and flowers)	99 g	12.4% (12.3 g)	Light green yellow foam

4.2.2 The effects of aqueous extracts from bogbean, geranium, meadowsweet and yarrow on cell viability

Aqueous extracts from bogbean, geranium, meadowsweet and yarrow, in concentrations from 0.1 up to100 µg/mL, did not affect viability of the DCs, as judged by microscope.

4.2.3 The effects of aqueous extracts from bogbean, geranium, meadowsweet and yarrow on expression of surface molecules on DCs

As observed with the ethanol extracts, culturing DCs in the presence of the of aqueous extracts from bogbean, geranium, meadowsweet and yarrow did not affect expression (percentage of positive cells or MFI) of CD14, CD86, CCR7, DC-SIGN or HLA-DR (appendix 2, figures A1-A4).

4.2.4 The effects of aqueous extracts from bogbean, geranium, meadowsweet and yarrow on cytokine secretion by DCs

Figures 10-12 show the effects of aqueous extracts from the four plants (0.1 to 100 µg/mL) on secretion of IL-12p40, IL-10, IL-6 and IL-23 by DCs. DCs matured in the presence of aqueous extracts from yarrow at the concentrations, 1, 10 and 50 µg/mL, secreted less IL-12p40 than DCs matured without plant extracts (figure 10). There was also a tendency towards less IL-12p40 secretion by DCs matured in the presence of aqueous extract from bogbean at all concentrations, compared with IL-12p40 secretion by DCs matured without plant extracts, but the difference was not statistically significant. Vitamin D3 decreased IL-12p40 secretion by DCs when compared with IL-12p40 secretion by DCs matured without vitamin D3 (figure 10).

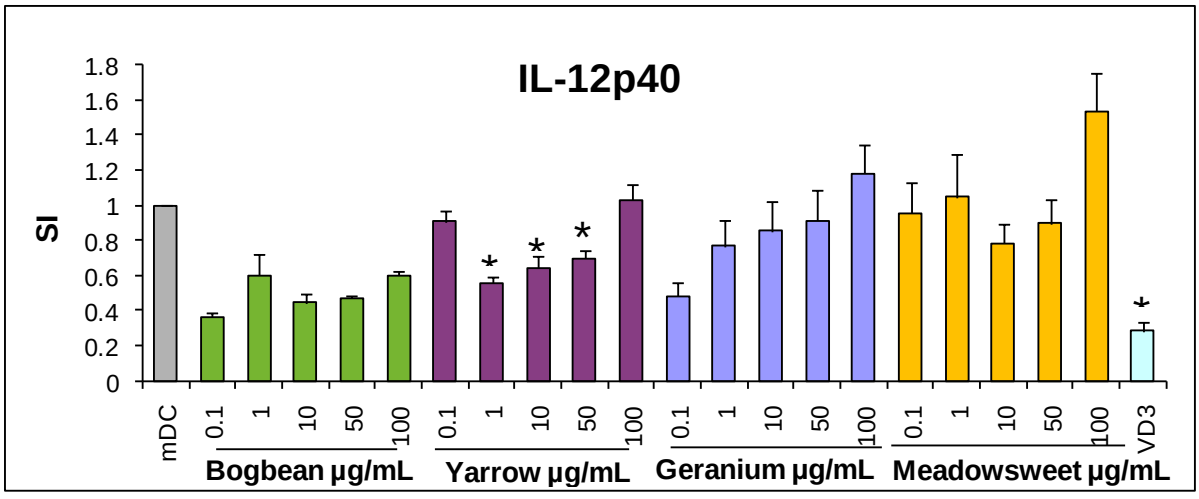

Figure 10. The effects of aqueous extracts from plants on IL-12p40 secretion by DCs.
DCs were matured without or in the presence of extracts from bogbean, geranium, meadowsweet and yarrow at concentrations from 0.1 to 100 µg/mL. Results are shown as SI ± SEM, n=3-4. mDC, mature dendritic cells; VD3, vitamin D3. * Different from DCs matured without plant extracts (mDC), $p<0.05$.

DCs matured in the presence of 50 or 100 µg/mL of aqueous extract from yarrow secreted more IL-10 than DCs matured without plant extracts (figure 11).

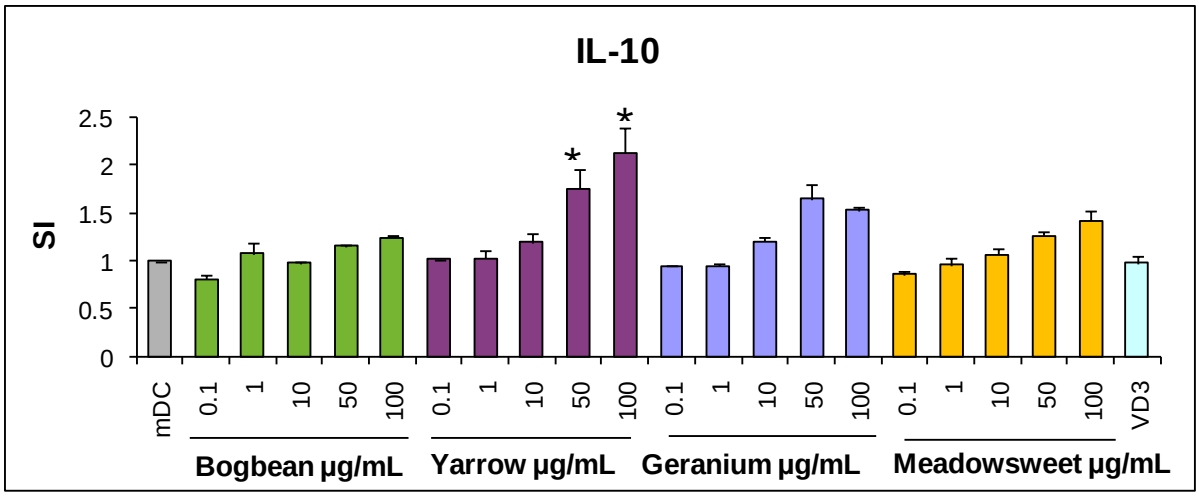

Figure 11. The effects of aqueous extracts from plants on IL-10 secretion by DCs.
DCs were matured without or in the presence of extracts from bogbean, geranium, meadowsweet and yarrow at concentrations from 0.1 to 100 µg/mL. Results are shown as SI ± SEM, n=3-4. mDC, mature dendritic cells; VD3, vitamin D3. * Different from DCs matured without plant extracts (mDC only), $p<0.05$.

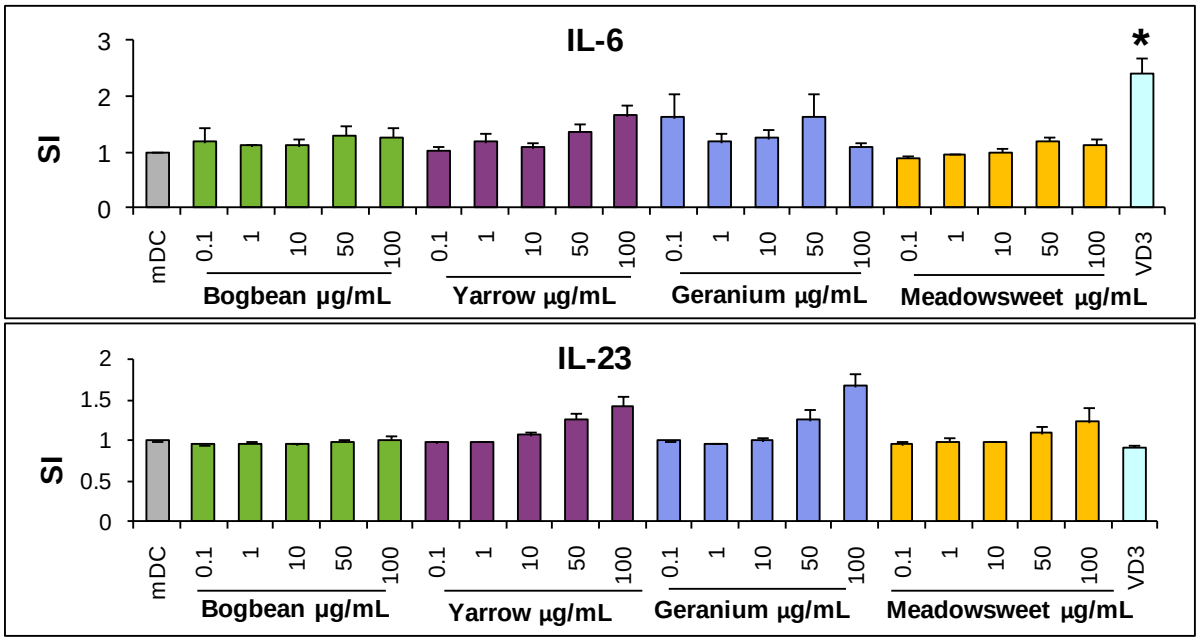

Figure 12. The effects of aqueous extracts from plants on IL-6 and IL-23 secretion by DCs.
DCs were matured without or in the presence of extracts from bogbean, yarrow, geranium and meadowsweet at concentrations from 0.1 to 100 µg/mL. Results are shown as SI ± SEM, n=3-4. mDC, mature dendritic cells; VD3, vitamin D3.

Aqueous extracts from the four plants did not affect DC secretion of IL-6 or IL-23 (figure 12). However, vitamin D3 increased the IL-6 secretion compared with DCs cultures without vitamin D3.

To assess if the effects of the aqueous extracts on cytokine secretion by DCs were pro- or anti-inflammatory, the ratio of secreted pro-inflammatory IL-12p40 to anti-inflammatory IL-10 was calculated. Maturing DCs in the presence of extracts from bogbean and yarrow led to a ratio of IL-12p40 to IL-10 that was below 1 (figure 13).

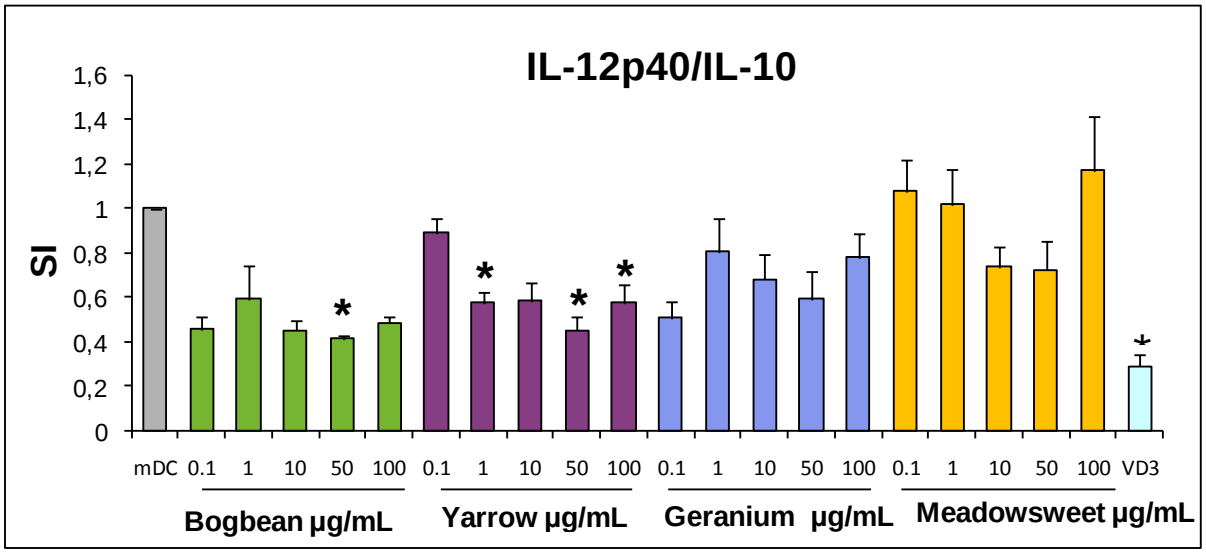

Figure 13. The effects of aqueous extracts from plants on the ratio of secreted IL-12p40 and IL-10 by DCs. DCs were matured without or in the presence of extracts from bogbean, yarrow, geranium and meadowsweet at concentrations from 0.1 to 100 µg/mL. Results are shown as the ratio of SI for IL-12p40 to SI for IL-10 ± SEM, n=3-4. mDC, mature dendritic cells; VD3, vitamin D3. * Different from DCs matured without plant extracts (mDC), $p<0.05$.

4.3 The effects of DCs matured in the presence of aqueous extracts from bogbean and yarrow on stimulation of allogeneic $CD4^+$ T cells

As the aqueous extracts from bogbean and yarrow affected cytokine secretion by DCs, the effects of DCs stimulated in the presence or absence of aqueous extracts from bogbean and yarrow on stimulation of allogeneic $CD4^+$ T cells was analysed.

4.3.1 Phenotype of and cytokine secretion by DCs matured in the presence of aqueous extracts from bogbean and yarrow after co-culture with allogeneic $CD4^+$ T cells

Aqueous extracts from bogbean and yarrow did not affect DC expression of CD86 or HLA-DR, (percentage of positive cells or MFI), after co-culture with allogeneic $CD4^+$ T cells (appendix, figure A5). DCs that had been matured in the presence of extract from bogbean (B-DCs) and then co-cultured with allogeneic $CD4^+$T cells, secreted less IL-12p40 than DCs that had been matured without plant extracts and then co-cultured with allogeneic $CD4^+$ T cells (figure 14). DCs that had been matured with extracts from yarrow (Y-DCs) and then co-cultured with allogeneic T cells secreted slightly more IL-12p40 than DCs matured without plant extracts, but the difference was not statistically

signif*i*cant (figure 14). There was no difference in secretion of IL-6 or IL-23 by DCs matured without or in the presence of plant extracts and co-cultured with allogeneic $CD4^+$ T cells (appendix, figure A6).

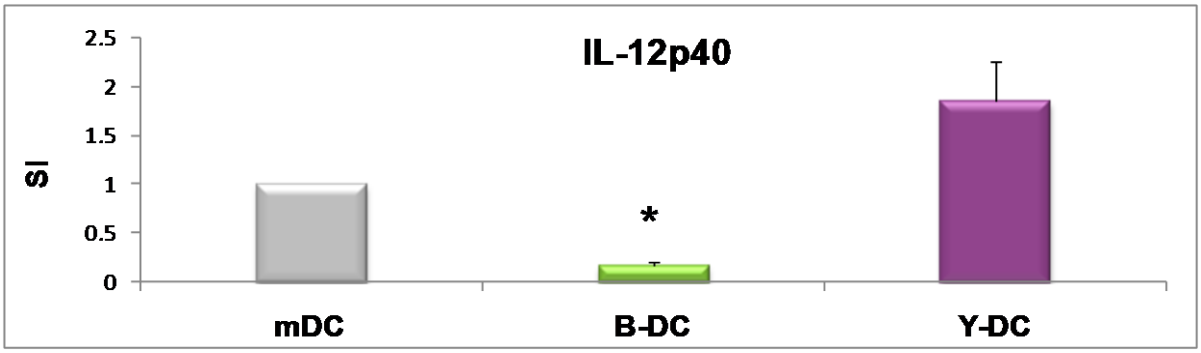

Figure 14. The effects of aqueous extracts from bogbean and yarrow on IL-12p40 secretion by DCs after co-culture with allogeneic $CD4^+$T cells.
DCs were matured without or in the presence of plant extracts at 50 µg/mL and then co-cultured with allogeneic $CD4^+$ T cells at a ratio of 1:10. Results are shown as SI ± SEM, n=12. mDC, mature dendritic cells; B-DC, DCs Matured in the presence of bogbean extract; Y-DC, DCs matured in the precence of yarrow extract. * Different from DCs matured without plant extracts (mDC), $p<0.05$.

4.3.2 Phenotype, cytokine secretion and proliferation of allogeneic $CD4^+$ T cells after co-culture with DCs matured in the presence of aqueous extracts from bogbean and yarrow

Co-culturing allogeneic $CD4^+$ T cells with B-DCs and Y-DCs did not affect their expression (percentage of positive cells or MFI) of CD4, CD25 or ICAM 1, compared with the expression of these molecules on allogeneic $CD4^+$ T cells that had been co-cultured with mDCs (appendix, figure A7).

Allogeneic $CD4^+$ T cells co-cultured with B-DCs secreted less IFN-γ than $CD4^+$ T cells co-cultured with mDCs (figure 8). There was no difference in IFN-γ secretion by allogeneic $CD4^+$ T cells co-cultured with Y-DCs or mDCs (figure 15).

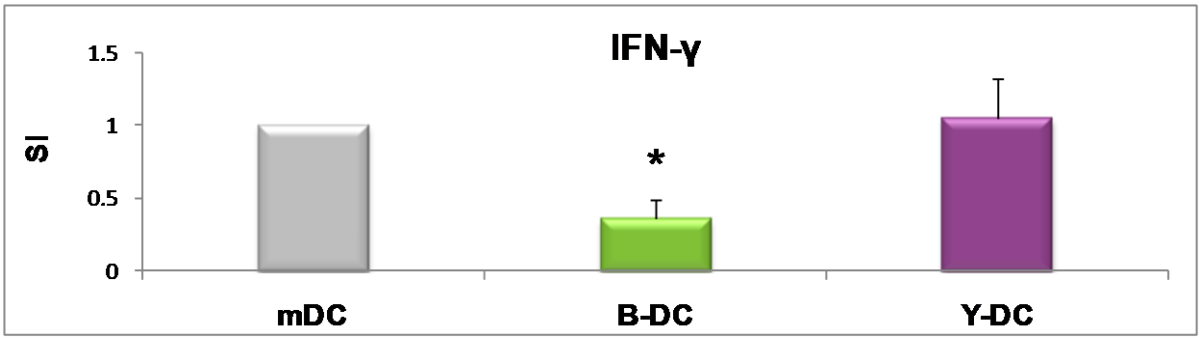

Figure 15. The effects of DCs matured in the presence of aqueous extracts from bogbean and yarrow on IFN-γ secretion by $CD4^+$ allogeneic T cells.
Allogeneic $CD4^+$ T cells were co-cultured with DCs that had been matured without or in the presence of plant extracts at 50 µg/mL, at a ratio of 10:1. Results are shown as SI ± SEM, n=12. mDC, mature dendritic cells; B-DC, DCs matured in the presence of bogbean extract; Y-DC, DCs matured with yarrow extract. * Different from allogeneic $CD4^+$ T cells co-cultured with DCs matured without plant extracts (mDC), $p<0.05$.

There was less IL-10 in supernatants from co-cultures of B-DCs and allogeneic $CD4^+$ T cells than in supernatants from co-culture of mDCs with $CD4^+$ T cells (figure 16). IL-10 levels in the supernatant from co-culture of Y-DCs and allogeneic $CD4^+$ T cells were not different from IL-10 levels in the supernatant from co-culture of mDCs and $CD4^+$ T cells (figure 16).

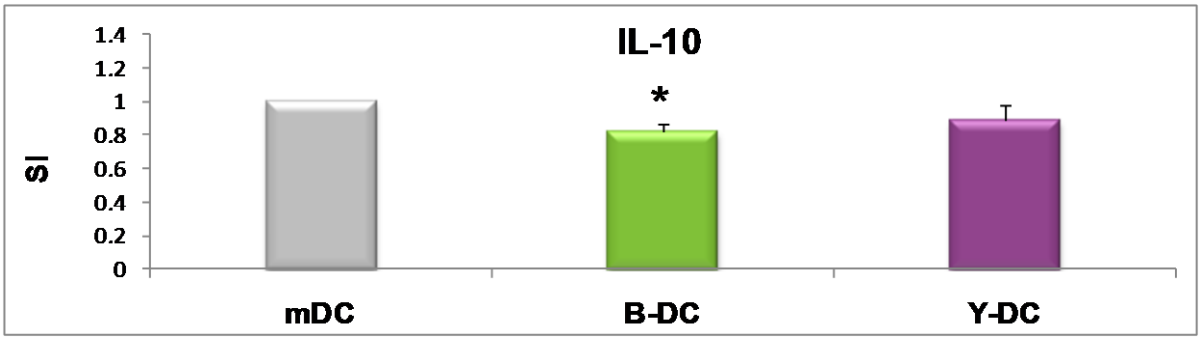

Figure 16. The effects of aqueous extracts from bogbean and yarrow on IL-10 levels in supernatants from co-cultures of DCs and allogeneic $CD4^+$ T cells.
DCs were matured without or in the presence of 50 µg/mL of plant extracts and then co-cultured with allogeneic $CD4^+$ T cells at a ratio of 1:10. Results are shown as SI ± SEM, n=12. mDC, mature dendritic cells; B-DC, DCs Matured in the presence of bogbean extract; Y-DC, DCs matured in the presence of yarrow extract. * Different from DCs matured without plant extracts (mDC), $p<0.05$.

Allogeneic $CD4^+$ T cells co-cultured with B-DCs or Y-DCs secreted less IL-17 than $CD4^+$ T cells co-cultured with mDCs (figure 17). No IL-4 production was detected in the supernatants following co-culture of DCs and allogeneic $CD4^+$ T cells.

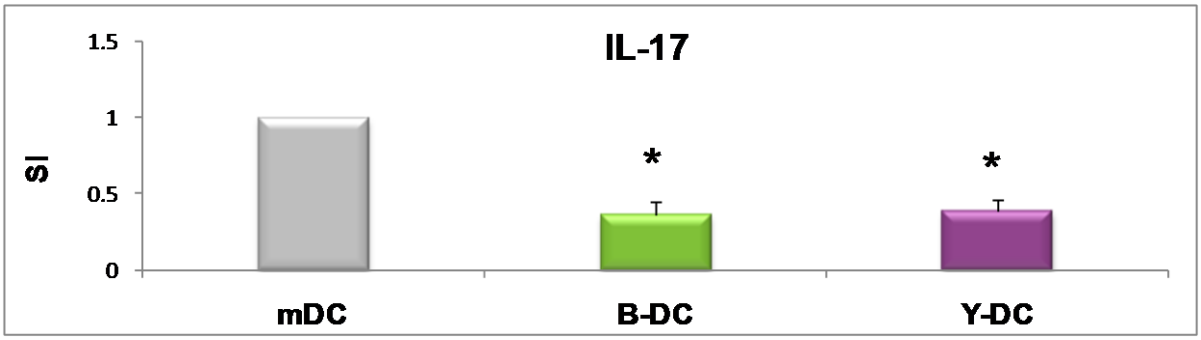

Figure 17. The effects of DCs matured in the presence of aqueous extracts from bogbean or yarrow on IL-17 secretion by $CD4^+$ allogeneic T cells.
Allogeneic $CD4^+$ T cells were co-cultured with DCs that had been matured without or in the presence of plant extracts at 50 µg/mL, at a ratio of 10:1. Results are shown as SI ± SEM, n=12. mDC, mature dendritic cells; B-DC, DCs matured in the presence of bogbean extract; Y-DC, DCs matured in the presence of yarrow extract. * Different from DCs matured without plant extracts (mDC), $p<0.05$.

There was a tendency towards less proliferation of allogeneic $CD4^+$ T cells when they were co-cultured with B-DCs or Y-DCs compared with their proliferation in co-cultures with mDCs, but the difference was not statistically significant (figure 18).

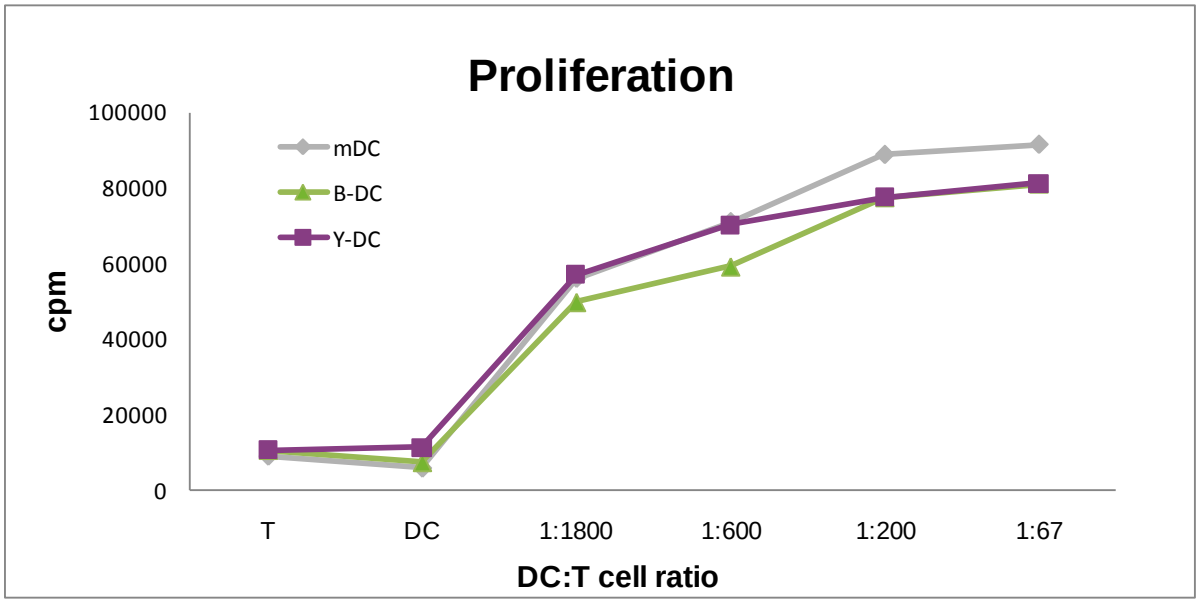

Figure 18. The effects of DCs matured in the presence of aqueous extracts from bogbean or yarrow on proliferation of allogeneic CD4$^+$ T cells.

Allogeneic CD4$^+$ T cells were co-cultured with DCs matured without or in the presence of plant extracts at 50 µg/mL, at various ratios. Results are shown as mean counts per minute (cpm), n= 8-10. mDC, mature dendritic cells; B-DC, DCs matured in the presence of bogbean extract; Y-DC, DCs matured in the presence of yarrow extract.

5 Discussion

The main results of the study show that aqueous extracts from bogbean and yarrow affected cytokine secretion by human DCs *in vitro*. The overall effect was anti-inflammatory as extracts from both plants led to a reduction in the ratio of secreted IL-12p40 over IL-10, when compared with that when DCs were matured in the absence of extracts. When DCs that had been matured in the presence of aqueous extract from bogbean were co-cultured with allogeneic $CD4^+$ T cells, the T cells secreted less IFN-γ and IL-17 than $CD4^+$ T cells co-cultured with DCs that had been matured without an extract. Allogeneic $CD4^+$ T cells co-cultured with DCs that had been matured with extracts from yarrow also secreted less IL-17 than $CD4^+$ T cells that had been co-cultured with DCs maturated without plant extracts.

In the study presented in this thesis a human DC model that was developed at the Centre for Rheumatology Research (Freysdottir*, et al.*, 2008) was used. In the model immature DCs were obtained by differentiating $CD14^+$ monocytes into DCs, which results in reduction of CD14 expression. Maturation of the DCs was confirmed by analyzing their expression of CD86 and HLA-DR, as these molecules are characteristic of mature DCs but very few immature DCs and monocytes express CD86 and HLA-DR (Murphy*, et al.*, 2008; Palucka*, et al.*, 1999). In the present study, expression of CD86 and HLA-DR, as measured by MFI, was low on monocytes and immature DCs, but increased when the DCs matured. These results are in accordance with previous results obtained using this *in vitro* DC model (Freysdottir*, et al.*, 2008).

The expression of several molecules that are thought to be present on mature DCs (CD86, CCR7, DC-SIGN and HLA-DR) and are involved in homing to lymph nodes and interaction with naïve T cells was analyzed following maturation of the DCs in the absence or presence of the plant extracts. Neither ethanol extracts nor aqueous extracts from bogbean, geranium, meadowsweet or yarrow affected expression of these surface molecules on DCs, indicating that DCs matured in the presence of these extracts may be equally capable of interacting with T cells as DCs matured in the absence of extracts. That the DCs matured in the presence of aqueous extracts from bogbean or yarrow were equally capable of interacting with T cells as DCs matured in the absence of extracts was confirmed, in part, by showing that proliferation of allogeneic $CD4^+$ T cells co-cultured with DCs that had been matured in the presence of aqueous extracts from either bogbean or yarrow was not affected. Furthermore, the expression of the activation molecules CD25 and ICAM-1 on allogeneic $CD4^+$ T cells was not affected.

In spite of the fact that the aqueous extracts from all four plants did not affect expression of surface molecules on DCs, they surely affected their cytokine secretion. Furthermore, aqueous extracts from both bogbean and yarrow affected the capacity of the DCs to influence cytokine secretion by allogeneic $CD4^+$ T cells. The decreased IFN-γ secretion by allogeneic $CD4^+$ T cells co-cultured with DCs matured in the presence of aqueous extract from bogbean, in the present study, may be due to the decreased IL-12p40 secreted by the DCs matured in the presence of the extract, as IL-12 has

been shown to induce IFN-γ production by Th1 cells (Murphy*, et al.*, 2008; Zhu*, et al.*, 2008) and to be necessary for optimal IFN-γ production (Kaplan *et al.*, 1996 ; Thierfelder *et al.*, 1996).

Interestingly the decreased IL-12p40 secretion by DCs matured in the presence of aqueous extract from yarrow was not seen following co-culture of the DCs with allogeneic $CD4^+$ T cells. In fact, DCs that had been matured in the presence of aqueous extract from yarrow and co-cultured with allogeneic $CD4^+$ T cells had a tendency towards more IL-12p40 secretion than DCs that had been matured in the absence of aqueous extract and co-cultured with allogeneic $CD4^+$ T cells. As IL-12 production is up-regulated by IFN-γ (Murphy*, et al.*, 2008), IL-12p40 secretion by DCs after co-culture with allogeneic T cells may be affected by the IFN-γ secreted by the T cells. Thus, the lack of effect of aqueous extract from yarrow on IL-12p40 secretion by DCs following co-culture with T cells may be due to the similar amount of IFN-γ present in co-cultures of T cells with DCs matured in the absence or presence of the extract. CD40L expressed by T cells that binds to CD40 on DCs may also play a role in the lack of effect of aqueous extract from yarrow on IL-12p40 secretion by DCs following co-culture with T cells, as *in vitro* experiments with stimulated human monocyte-derived DCs rapidly produced high levels of IL-12 upon CD40L stimulation (Giacomini*, et al.*, 2001; Vieira *et al.*, 2000). Finally, it is possible that the yarrow extract has to be present in the culture medium to affect IL-12p40 secretion by DCs but the extract is not present in the co-cultures.

Although IL-12p40 is a subunit present in IL-23 as well as IL-12 (Miossec*, et al.*, 2009) the plant extracts did not affect IL-23 levels in the culture medium from DCs and thus their effects on IL-12p40 are considered to be on IL-12.

The increase in IL-10 secretion observed for DCs when maturated in the presence of aqueous extract from yarrow was not observed when the DCs had been co-cultured with the allogeneic $CD4^+$ T cells. In contrast, supernatant from co-cultures of allogeneic $CD4^+$ T cells and DCs that had been matured in the presence of aqueous extract from bogbean had lower IL-10 levels than supernatant from co-cultures of allogeneic $CD4^+$ T cells and DCs that had been matured without extract. As both DCs and T cells are capable of secreting IL-10, it is impossible to determine whether the IL-10 measured in the supernatant from the co-cultures of DCs and allogeneic $CD4^+$ T cells is derived from the DCs or the T cells, making all interpretations of the data difficult. Therefore, the decreased IL-10 observed in co-cultures of B-DCs and $CD4^+$ T cells may indicate that the B-DCs, when stimulated with T cells, are secreting less IL-10 than mDCs or that the allogeneic $CD4^+$ T cells are secreting less IL-10 when co-cultured with B-DCs compared with mDCs. When the $CD4^+$ T cells and the DCs were cultured separately both cell types secreted very little IL-10 (data not shown), indicating that the IL-10 secretion by either DCs and/or T cells is dependent on stimulation. To find out whether the DCs or the T cells are responsible for the IL-10 production in the co-cultures, intracellular staining for IL-10 in conjunction with markers specific for T cells or DCs could be used.

Maturing DCs in the presence of either aqueous extract from bogbean or yarrow decreased IL-17 secretion by allogeneic $CD4^+$ T cells in the co-cultures. It is not clear how this effect is mediated. IL-23 and IL-6 are considered to influence differentiation and maintenance of human Th17 cells (Romagnani, 2008; Tesmer*, et al.*, 2008; Zhu*, et al.*, 2008) but maturing DCs in the presence of aqueous extract from bogbean or yarrow did not affect secretion of these cytokines indicating that these cytokines are not involved in mediating the decreased IL-17 secretion by T cells co-cultured with DCs matured in the presence of extracts from these plants. Other cytokines that may mediate the effects of the extracts are IL-1β, IL-21 and IL-22 which have all been linked to human Th17 differentiation and maintenance (Romagnani, 2008; Tesmer*, et al.*, 2008; Zhu*, et al.*, 2008). However, these cytokines were not measured in this study.

IL-17 is considered to have strong pro-inflammatory activity and to be a potent mediator of inflammatory responses in various tissues (Fujino *et al.*, 2003; Murphy*, et al.*, 2008). It has been implicated in several inflammatory disorders and may be a critical pathogenic factor in autoimmune inflammatory diseases, such as rheumatoid arthritis, multiple sclerosis, inflammatory bowel diseases and asthma, where increased IL-17 levels are observed (Fujino*, et al.*, 2003; Hofstetter *et al.*, 2005; Shahrara *et al.*, 2008; Zhao *et al.*, 2010). In addition, results from several studies using animal models of rheumatoid arthritis have suggested that treatments designed to block IL-17 may be beneficial (Lubberts *et al.*, 2005). Blocking IL-21 has also recently been shown to be effective in treating rheumatoid arthritis (Niu *et al.*, 2010) and multiple sclerosis (Nurieva *et al.*, 2007), but IL-21 is made by Th17 cells and is believed to be a stimulatory factor for Th17 differentiation and play a critical role in a positive feedback loop which facilitates induction of Th17 cells (Korn *et al.*, 2007; Niu *et al.*, 2010; Nurieva *et al.*, 2007). IL-22 is also produced by Th17 cells (Zheng *et al.*, 2007) and is increased in patients with rheumatoid arthritis (Ikeuchi *et al.*, 2005) and psoriasis (Caproni *et al.*, 2009). IL-1β, is however, produced by DCs and is over-expressed in human rheumatoid arthritis and has a well-established role in the disease pathology (Tesmer*, et al.*, 2008), suggesting that a change in IL-1 β may have affected the IL-17 secretion by the allogeneic $CD4^+$ T cells in this study.

In the present study DCs matured in the presence of aqueous extracts from bogbean and yarrow lead to decreased IL-17 secretion by allogeneic $CD4^+$ T cells when the cells were co-cultured. Thus, compounds isolated from bogbean and yarrow could be good candidates for natural products or lead compounds inhibiting autoimmune inflammatory diseases. However, Th17 cells are important as a protection against extracellular infections, e.g. extracellular bacteria and fungi (Happel *et al.*, 2003). Therefore, the isolated compound(s) from bogbean and yarrow may be harmful for individuals with infections where strong immune response against extracellular infections is needed.

Several constituents have been isolated from bogbean, yarrow, geranium and meadowsweet, but only a few of those have been tested for their effects on the immune system and no data exist on the effects of these plants on DCs. Different parts of these plants have been used in folk medicine, and therefore in the first part of the study, when testing the effects of ethanol extracts from the plants,

various parts from the plants were tested (root, stalk, leaves and flowers). None of these, whether used alone or in combination, had any effect on maturation of the DCs (data not shown) suggesting that ethanol extract of any part of these plants does not affect DCs. Culturing DCs with ethanol extracts, at concentrations of 50 and 100 µg/mL, from all the four plants, led to cell death. Cell death was not due to the solvent, as DMSO in the same concentration as used to dissolve the material from the plant extracts, did not affect cell viability. It would be interesting to investigate which constituent(s) in the plants had this effect and whether the DCs were dying because of apoptosis or necrosis. Furthermore, it would be interesting to determine whether these constituents would affect other cells than DCs in culture, e.g. cancer cells, as it is known that many cancer drugs originate from natural products can (Wang*, et al.*, 2007).

The aqueous extracts used in this study contain compounds that are water soluble. As the ethanol extract was not effective in inducing immunological effects, the active compound(s) are likely to be water soluble constituents, like polysaccharides, glycosides, polypeptides or lectins (Samy*, et al.*, 2010). Polysaccharides, such as pectins, from various plants are reported to have effects on the immune system (Samuelsen*, et al.*, 1996; The Review of Natural Products, 2009). Therefore, it would be interesting to perform bioguided isolation of polysaccharides from the aqueous extracts from bogbean and yarrow, and that will be part of future studies.

6 Conclusion

Aqueous extracts from bogbean and yarrow induce a tolerogenic-like phenotype of DCs. When these DCs are used as antigen presenting cells *in vitro*, they have reduced capacity to induce Th17 stimulation of allogeneic $CD4^+$ T cells and bogbean extract also reduced the capacity of DCs to induce a Th1 response. These results indicate that both extracts contain compound(s) that induce DCs to stimulate an anti-inflammatory phenotype of T cells *in vitro*.

As both Th1 and Th17 cells may be pathogenic and play a role in autoimmune diseases, these results indicate that aqueous extracts from yarrow and bogbean contain compound(s) that may reduce autoimmune responses by affecting DCs and their interaction with $CD4^+$ T cells.

The active compound(s) in the aqueous extracts from bogbean and yarrow need to be identified, using bioguided isolation, and further research is needed to confirm the effects of the extracts or the isolated compound(s) *in vivo*, e.g. in a rat model of arthritis.

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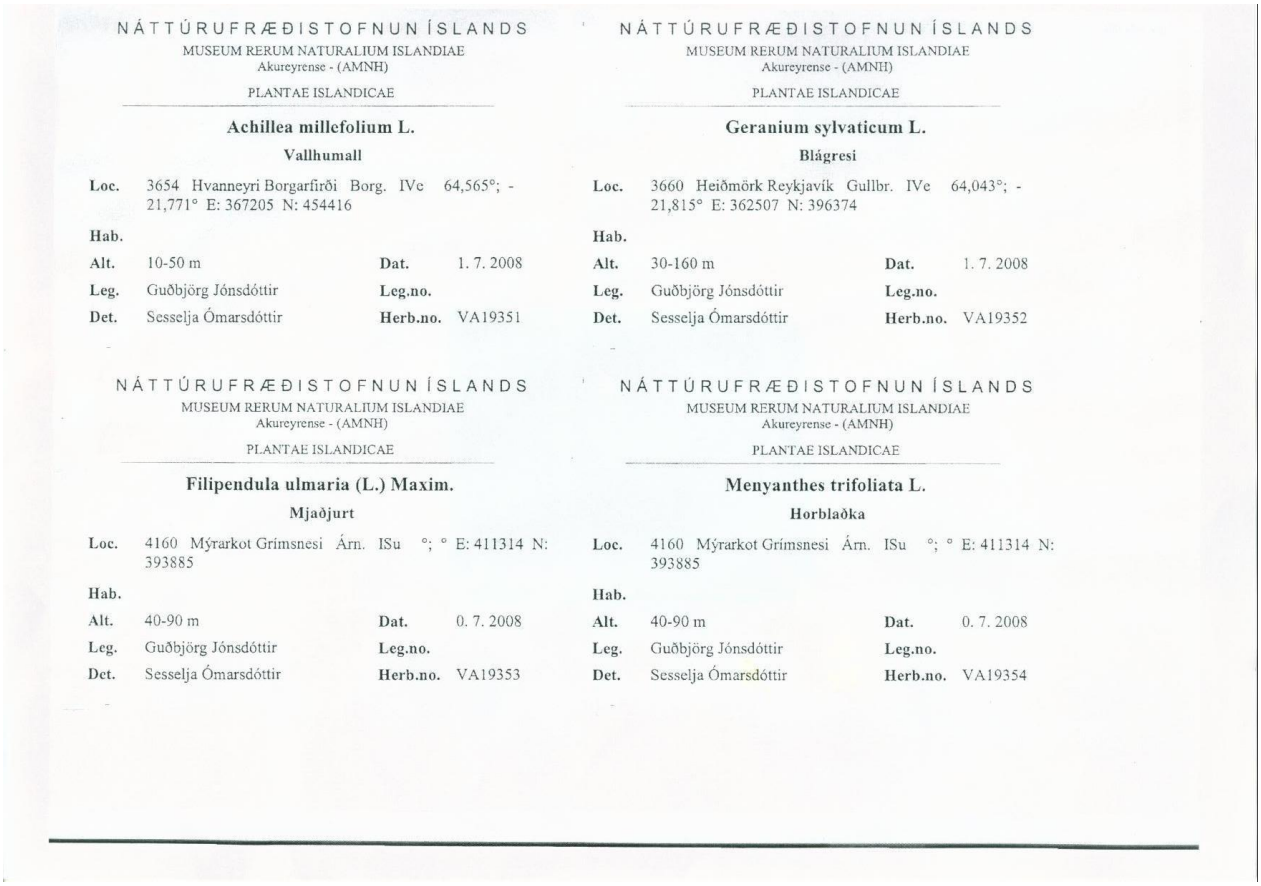
Appendix

Appendix 1

Document 1. Voucher specimens from The Icelandic Institute of Natural History, Akureyri.



NÁTTÚRUFRÆÐISTOFNUN ÍSLANDS
MUSEUM RERUM NATURALIUM ISLANDIAE
Akureyrense - (AMNH)
PLANTAE ISLANDICAE

Achillea millefolium L.

Vallhumall

Loc. 3654 Hvanneyri Borgarfirði Borg. IVe 64,565°; -21,771° E: 367205 N: 454416
Hab.
Alt. 10-50 m **Dat.** 1. 7. 2008
Leg. Guðbjörg Jónsdóttir **Leg.no.**
Det. Sesselja Ómarsdóttir **Herb.no.** VA19351

NÁTTÚRUFRÆÐISTOFNUN ÍSLANDS
MUSEUM RERUM NATURALIUM ISLANDIAE
Akureyrense - (AMNH)
PLANTAE ISLANDICAE

Geranium sylvaticum L.

Blágresi

Loc. 3660 Heiðmörk Reykjavík Gullbr. IVe 64,043°; -21,815° E: 362507 N: 396374
Hab.
Alt. 30-160 m **Dat.** 1. 7. 2008
Leg. Guðbjörg Jónsdóttir **Leg.no.**
Det. Sesselja Ómarsdóttir **Herb.no.** VA19352

NÁTTÚRUFRÆÐISTOFNUN ÍSLANDS
MUSEUM RERUM NATURALIUM ISLANDIAE
Akureyrense - (AMNH)
PLANTAE ISLANDICAE

Filipendula ulmaria (L.) Maxim.

Mjaðjurt

Loc. 4160 Mýrarkot Grímsnesi Árn. ISu °; ° E: 411314 N: 393885
Hab.
Alt. 40-90 m **Dat.** 0. 7. 2008
Leg. Guðbjörg Jónsdóttir **Leg.no.**
Det. Sesselja Ómarsdóttir **Herb.no.** VA19353

NÁTTÚRUFRÆÐISTOFNUN ÍSLANDS
MUSEUM RERUM NATURALIUM ISLANDIAE
Akureyrense - (AMNH)
PLANTAE ISLANDICAE

Menyanthes trifoliata L.

Horblaðka

Loc. 4160 Mýrarkot Grímsnesi Árn. ISu °; ° E: 411314 N: 393885
Hab.
Alt. 40-90 m **Dat.** 0. 7. 2008
Leg. Guðbjörg Jónsdóttir **Leg.no.**
Det. Sesselja Ómarsdóttir **Herb.no.** VA19354

Appendix 2

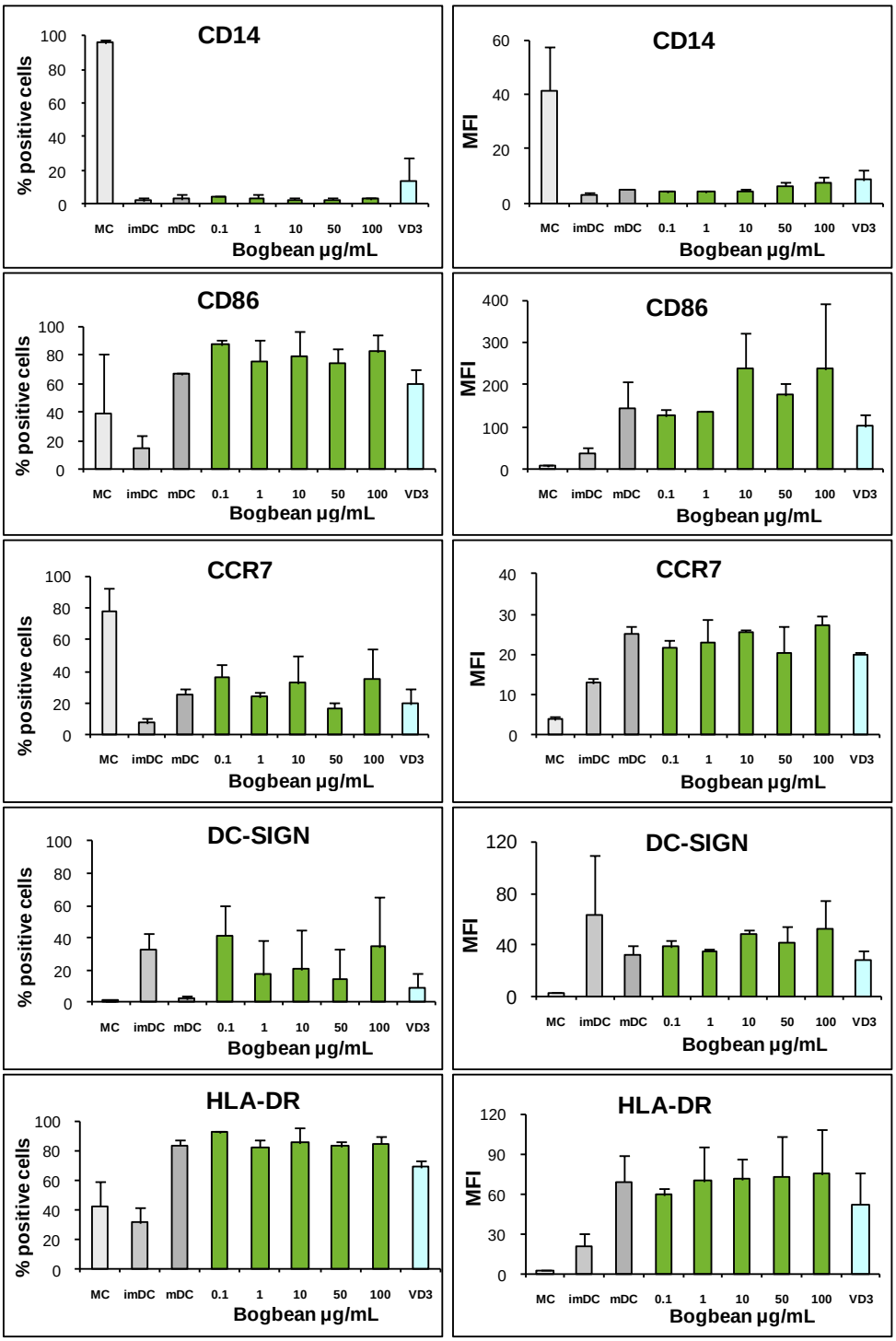


Figure A1. Effects of aqueous extract from bogbean on the proportion of cells expressing CD14, CD86, CCR7, DC-SIGN and HLA-DR and mean fluorescence intensity of expression of these molecules. DCs were matured without or in the presence of aqueous extract from bogbean at concentrations from 0.1 to 100 µg/mL. Results are shown as mean percentage of positive cells or MFI ± SEM, n=2-3. MC, monocytes; imDC, immature dendritic cells; mDC, mature dendritic cells; VD3, vitamin D3.

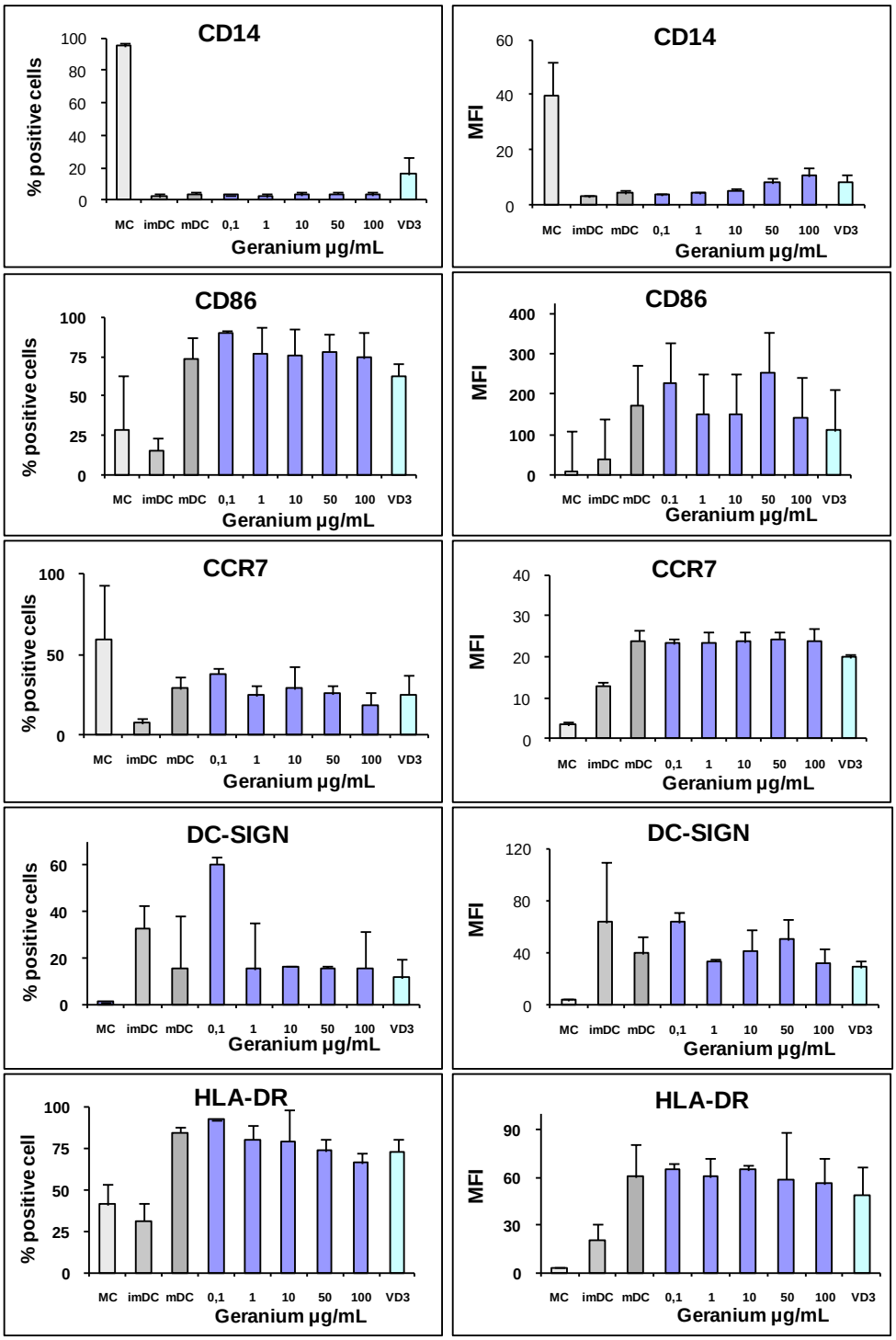


Figure A2. The effects of aqueous extract from geranium on the proportion of cells expressing CD14, CD86, CCR7, DC-SIGN and HLA-DR and mean fluorescence intensity of expression of these molecules. DCs were matured without or in the presence of aqueous extract from geranium at concentrations from 0.1 to 100 µg/mL. Results are shown as mean percentage of positive cells or MFI ± SEM, n=2-3. MC, monocytes; imDC, immature dendritic cells; mDC, mature dendritic cells; VD3, vitamin D3.

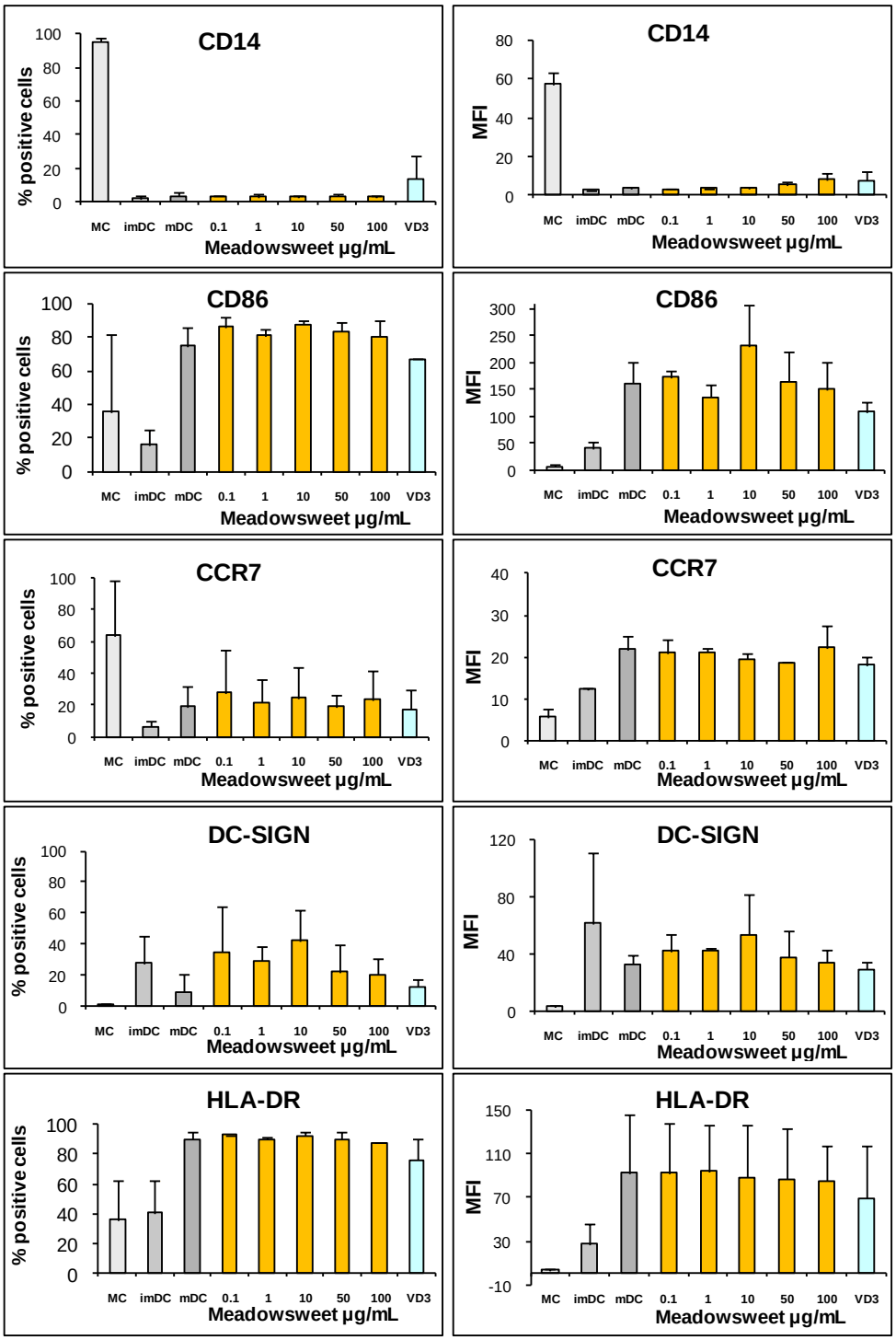


Figure A3. Effects of aqueous extract from meadowsweet on the proportion of cells expressing CD14, CD86, CCR7, DC-SIGN and HLA-DR and mean fluorescence intensity of expression of these molecules. DCs were matured without or in the presence of aqueous extract from bogbean at concentrations from 0.1 to 100 µg/mL. Results are shown as mean percentage of positive cells or MFI ± SEM, n=2. MC, monocytes; imDC, immature dendritic cells; mDC, mature dendritic cells; VD3, vitamin.

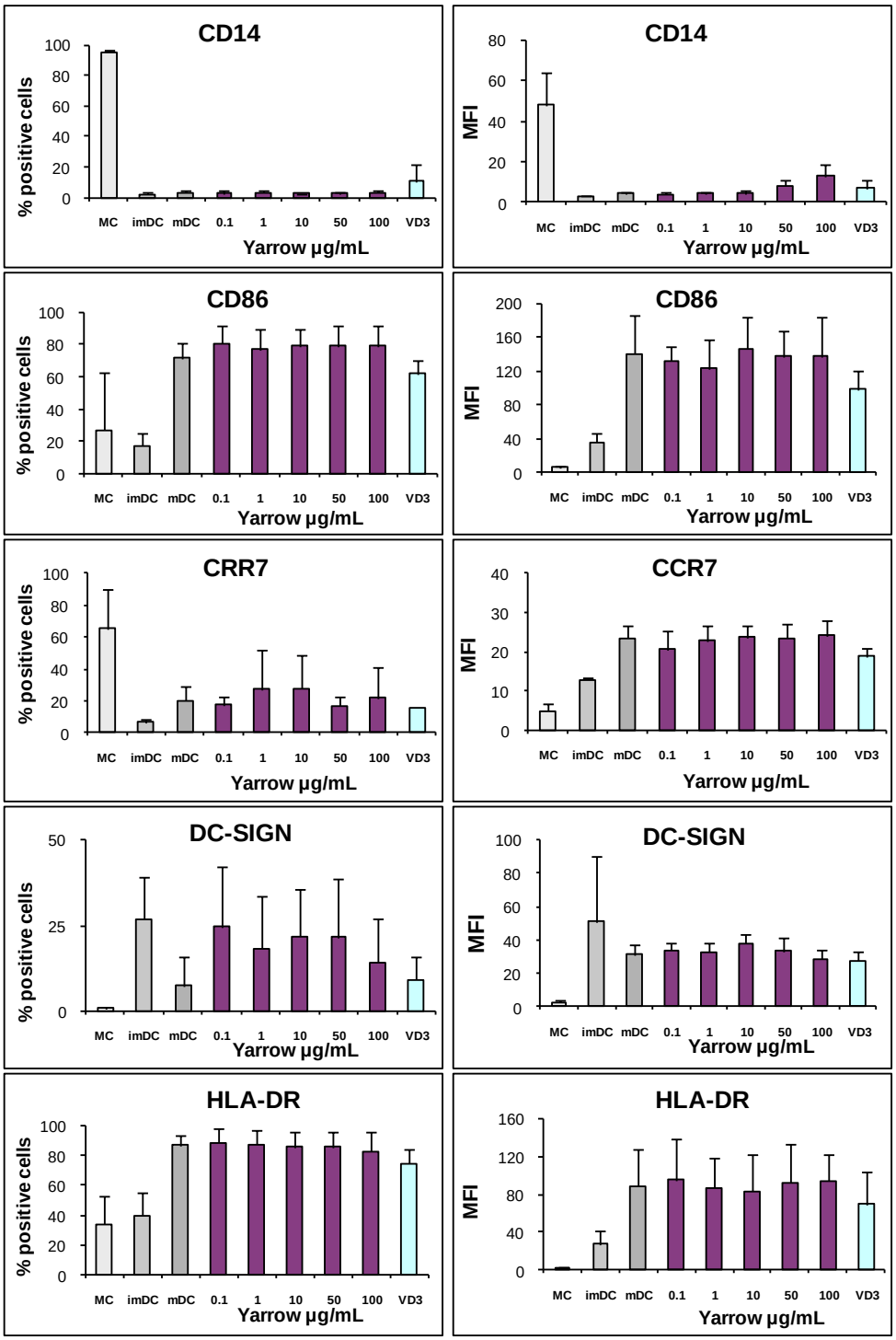


Figure A4. The effects of aqueous extract from yarrow on the proportion of cells expressing CD14, CD86, CCR7, DC-SIGN and HLA-DR and mean fluorescence intensity of expression of these molecules. DCs were matured without or in the presence of aqueous extracts from bogbean at concentrations from 0.1 to 100 µg/mL. Results are shown as mean percentage of positive cells or MFI ± SEM, n=3. MC, monocytes; imDC, immature dendritic cells; mDC, mature dendritic cells; VD3, vitamin D3.

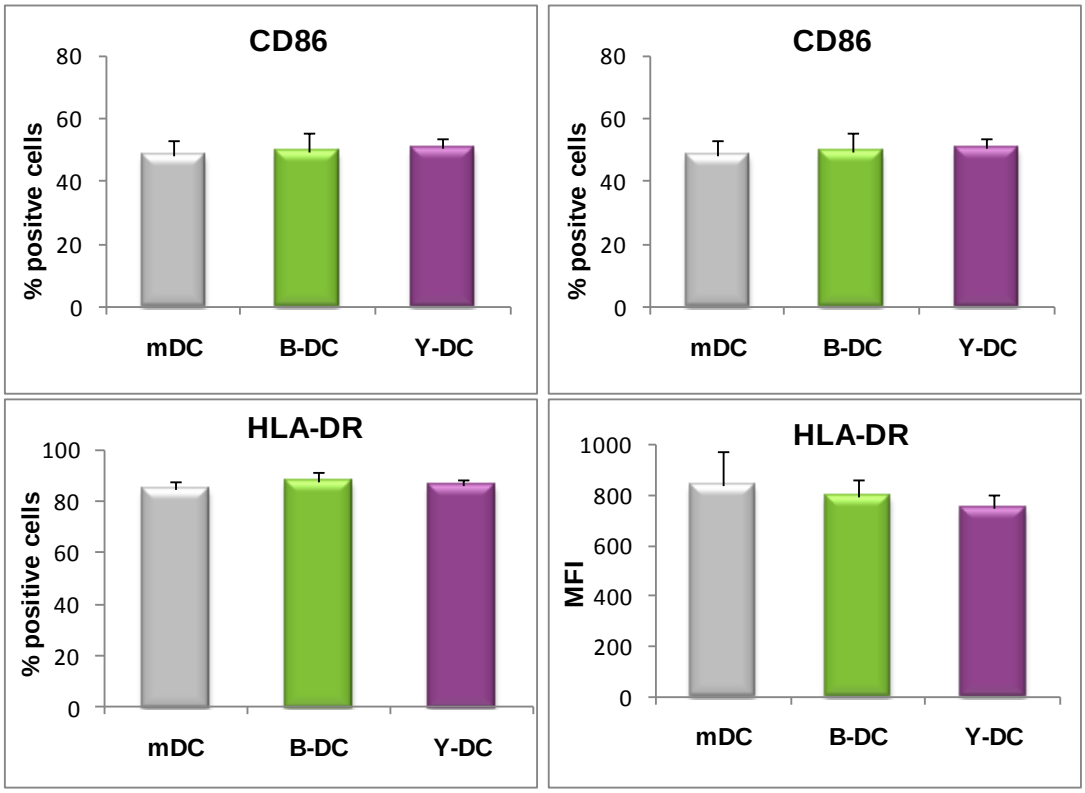


Figure A5. The effects of aqueous extracts from bogbean and yarrow on the proportion of DCs expressing CD86 and HLA-DR and mean fluorescence intensity of CD86 and HLA-DR expression on DCs after co-culture with allogeneic CD4$^+$ T cells. DCs were matured without or in the presence of plant extracts at 50 µg/mL and then co-cultured with allogeneic CD4$^+$ T cells at the ratio 1:10. Results are shown as mean percentage of positive cells or MFI ± SEM, n=4. mDC, mature dendritic cells; B-DC, DCs matured with bogbean extract; Y-DC, DCs matured with yarrow extract.

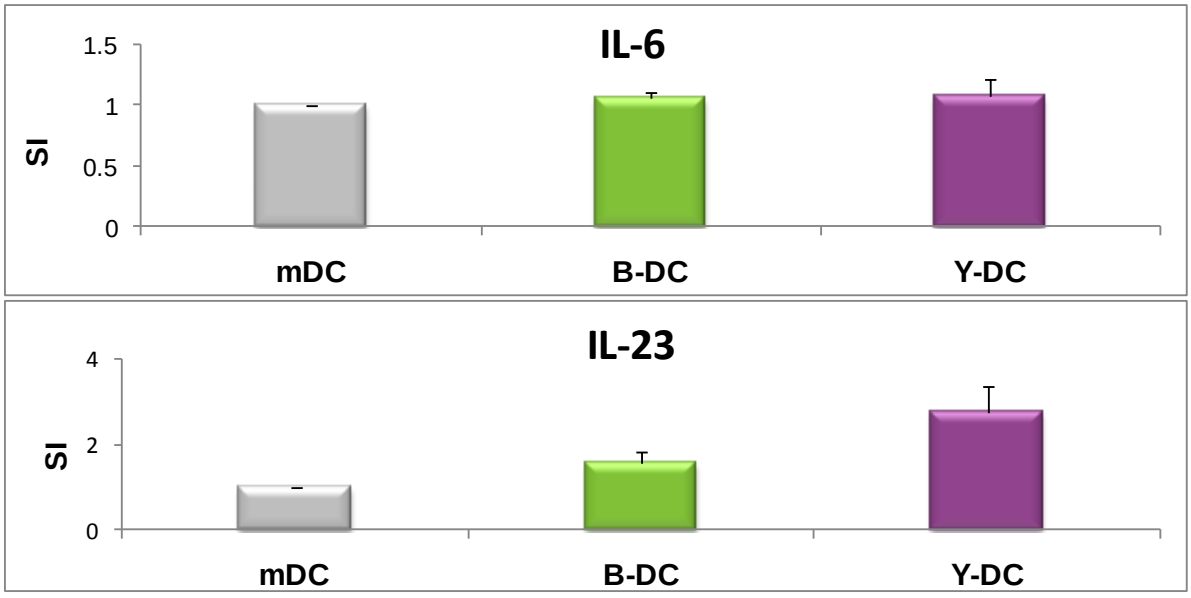


Figure A6. The effects of aqueous extracts from bogbean and yarrow on IL- 6 and IL-23 secretion by DCs after co-culture with allogeneic CD4$^+$ T cells. DCs were matured without or in the presence of plant extracts at 50 µg/mL and then co-cultured with allogeneic CD4+ T cells at a ratio of 1:10. Results are shown as SI ± SEM, n=12. mDC, mature dendritic cells; B-DC, DCs matured with bogbean extract; Y-DC, DCs matured in the presence of yarrow extract.

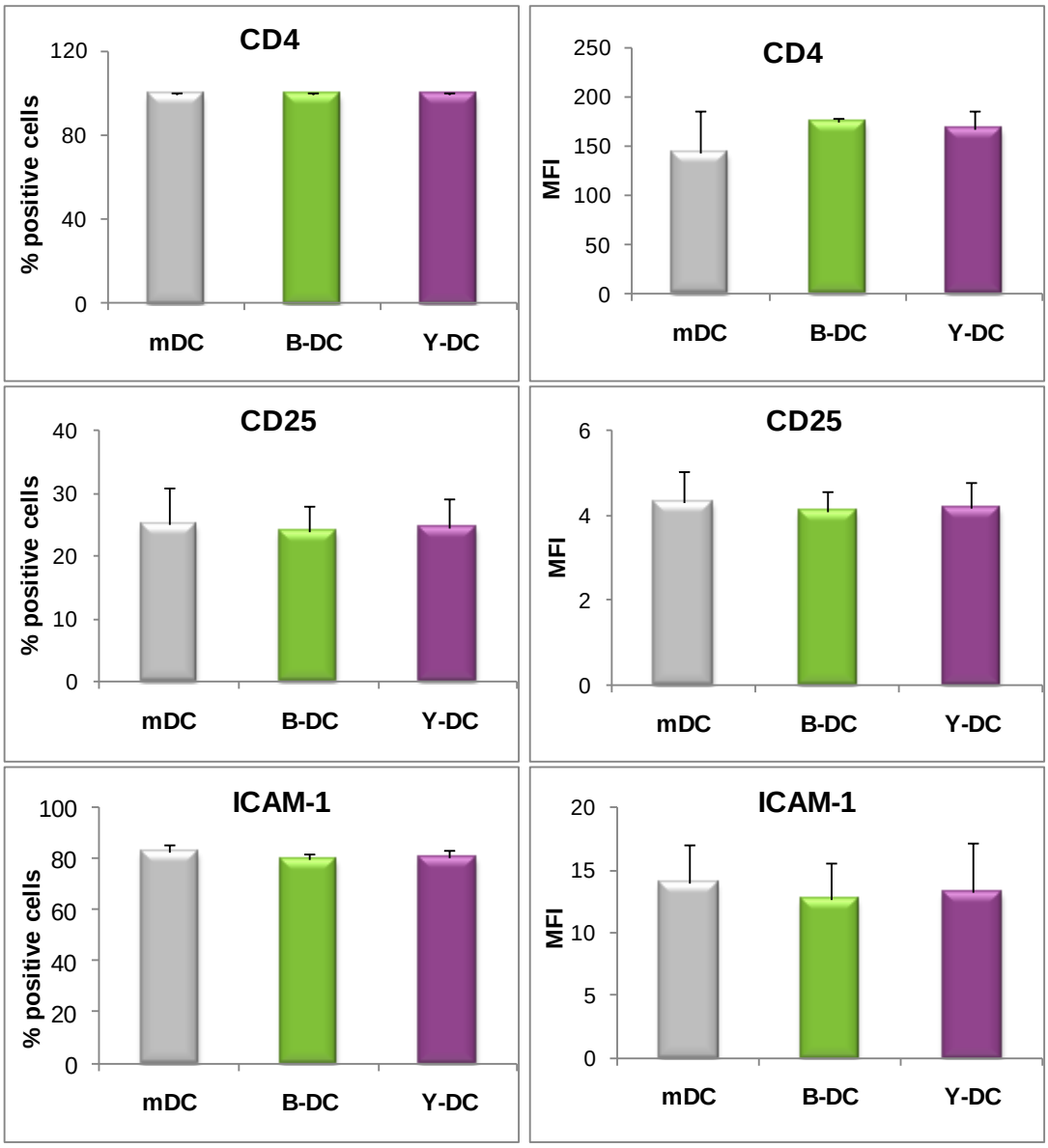


Figure A7. The effects of aqueous extracts from bogbean and yarrow on the proportion of T cells expressing CD4, CD25 and ICAM-1 and mean fluorescence intensity of CD4, CD25 and ICAM-1 expression by allogeneic CD4$^+$ T cells. DCs were matured without or in the presence of plant extracts at 50 µg/mL and then co-cultured with allogeneic CD4$^+$ T cells at a ratio of 1:10. Results are shown as mean percentage of positive cells or MFI ± SEM, n=4. mDC, mature dendritic cells; B-DC, DCs matured with bogbean extract; Y-DC, DCs matured with yarrow extract.