

# Characterisation of a Novel Tick-derived Dendritic Cell Modulator: Japanin

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by

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## Abstract

Dendritic cells (DC) play a key role in immunity and represent a great target for modulation, because of their ability to prime T cells and direct their polarisation into effector subsets. Ticks release immunomodulatory compounds in their saliva, possibly in order to evade host immune responses during feeding. We have recently reported that *Rhipicephalus appendiculatus* ticks produce 'Japanin', a secretory lipocalin that arrests differentiation of monocytes into DC and reprogrammes maturation of DC in response to various stimuli towards a tolerogenic phenotype\*. Japanin was cloned and recombinantly expressed in a baculovirus system for subsequent immunological and biochemical analysis. This study was set out to further investigate the immunomodulatory activity of Japanin as well as the underlying mechanism of action. We have discovered that Japanin prevents DC-mediated proliferation and polarisation of allogeneic T cells. Experiments with labelled Japanin have demonstrated that it binds predominantly to *ex vivo* generated human monocyte-derived DC (moDC) and to a reduced degree to monocyte and DC populations in peripheral blood, yet to no other blood leucocytes. We have identified CD206, also known as the mannose receptor, as a Japanin-binding receptor on moDC. This identification has been achieved by crosslinking and subsequent pull-down of Japanin-receptor complexes from moDC. Affinity studies with recombinant CD206 constructs have confirmed the binding to Japanin. Moreover, the binding has been verified by specific siRNA knock-down of CD206 in moDC, which resulted in significantly decreased binding of Japanin. Unexpectedly, CD206 has appeared to be dispensable for at least most of the DC-modulatory activity of Japanin. Therefore, attempts were made to determine other factors in the mode of action of Japanin, through which we have found that IL-10 is not essentially involved. Further results have suggested that the activity of Japanin demands cell contact. Collectively, we have come to the conclusion that the mechanism of action of Japanin might require internalisation by DC, potentially enabling modulation of intracellular pathways involved in the regulation of DC maturation.

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## Abbreviations

2ME	2-mercaptoethanol
ABIN-1	A20 binding and inhibitor of NF- $\kappa$ B1
ABTS	2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
AIRE	Autoimmune regulator
APC	Antigen-presenting cells
B7-H1	B7 homolog 1
BDCA	Blood dendritic cell antigen
BFA	Brefeldin A
BMDC	Bone marrow-derived dendritic cell(s)
BMM	Bone marrow-derived macrophage(s)
BS <sup>3</sup>	Bissulfosuccinimidyl suberate
BSA	Bovine serum albumin
BST2	Bone marrow stromal Ag 2
Ca <sup>2+</sup>	Calcium ion
CCR	C-C chemokine receptor
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CFSE	Carboxyfluorescein succinimidyl ester
CLEC7A	C-type lectin domain family 7 member A
CLEC9A	C-type lectin domain family 9 member A
CLEVER-1	Common lymphatic endothelial and vascular endothelial receptor-1
Con A	Concanavalin A
COX	Cyclo-oxygenase
CR	Cysteine-rich
CREB	Cyclic-AMP-responsive-element-binding protein
CR-FNII	Cysteine-rich fibronectin type II
CTLA4	Cytotoxic T lymphocyte antigen 4
CTLD	C-type lectin-like domain
CXCL	CXC chemokine ligand
CXCR	CXC chemokine receptor
Da	Dalton
DAMP	Damage-associated molecular pattern
DC	Dendritic cell(s)
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DMEM	Dulbecco's Modified Eagle's Medium
DN	Double negative
DNA	Deoxyribonucleic acid
DTH	Delayed-type hypersensitivity

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DTT	Dithiothreitol
DXM	Dexamethasone
EAE	Experimental autoimmune encephalitis
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FCS	Foetal calf serum
Flt3L	FMS-like tyrosine kinase 3 ligand
FNII	Fibronectin type II
FOXP3	Forkhead box P3
GALT	Gut-associated lymphoid tissues
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HEV	High endothelial venule
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
IC-ALI	Immune complex-induced acute lung injury
ICAM-1	Intracellular adhesion molecule-1
ICOS	Inducible T cell co-stimulator
ICOSL	Inducible T cell co-stimulator ligand
iDC	Interstitial dendritic cell(s)
IDO	Indoleamine-2,3-dioxygenase
IFN	Interferon
IL	Interleukin
IL-10R	Interleukin-10 receptor
IRF	Interferon regulatory factor
ITB2	Integrin $\beta$ 2
iTreg	Inducible FoxP3 <sup>+</sup> regulatory T
IU	International unit
K <sub>D</sub>	Dissociation constant
KO	Knock-out
LAT	Linker for activation of T cells
LB	Luria Broth
LBP	Lipopolysaccharide-binding protein
LC	Langerhans cell
LCA	Leucocyte common antigen
Lck	Lymphocyte-specific protein tyrosine kinase
L-Gln	L-glutamine
LIR	Lipocalins from <i>Ixodes ricinus</i>
L-PGDS	Lipocalin-type prostaglandin D synthase

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LPS	Lipopolysaccharide
LTB <sub>4</sub>	Leukotriene B <sub>4</sub>
MAL	MyD88-adaptor-like protein
MAPK	Mitogen-activated protein kinase
M-CSF	Macrophage colony-stimulating factor
MD-2	Myeloid differentiation factor 2
MEK	Mitogen-activated protein kinase kinase
MHC	Major histocompatibility complex
MFI	Mean fluorescence intensity
MLR	Mixed leucocyte reaction
MLR	Mixed leucocyte reaction
moDC	Monocyte-derived dendritic cell(s)
moi	Multiplicity of infection
MRC1	Macrophage mannose receptor 1
MS	Multiple sclerosis
mTEC	Medullary thymic epithelial cells
NFAT	Nuclear factor of activated T cells
NF- $\kappa$ B	Nuclear factor $\kappa$ -light-chain-enhancer of activated B cells
NK	Natural killer
NLR	Nucleotide-binding oligomerisation domain-like receptor
nTreg	Natural regulatory T
OD	Optical density
Osp	Outer surface protein
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline plus Tween 20
PD-1	Programmed cell death protein 1
pDC	Plasmacytoid DC(s)
PD-L1	Programmed death ligand 1
PEG	Polyethylene glycol
pfu	Plaque-forming unit
PG	Prostaglandin
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate
PMSF	Phenylmethanesulfonylfluoride
PNGase F	Peptide:N-Glycosidase F
Poly I:C	Polyinosinic:polycytidylic acid
Pre-cDC	Precursors of conventional dendritic cells
PRR	Pattern recognition receptor

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pSTAT3	Phosphorylated signal transducer and activator of transcription 3
RA	Retinoic acid
RACE-PCR	Rapid amplification of cDNA ends with polymerase chain reaction
Ra-HBP	<i>Rhipicephalus appendiculatus</i> -derived histamine-binding protein
RANK	Receptor activator of nuclear factor- $\kappa$ B
RANKL	Receptor activator of nuclear factor- $\kappa$ B ligand
RIG-I	Retinoic acid-inducible gene I
RNA	Ribonucleic acid
RPMI	Named after Roswell Park Memorial Institute
RT-PCR	Reverse transcriptase polymerase chain reaction
RU	Response units
Scr	Sarcoma
SCS	Subcapsular sinus
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	Size-exclusion chromatography
SGE	Salivary gland extract
Siglec-H	Sialic acid-binding immunoglobulin-like lectin H
siRNA	Small interfering ribonucleic acid
SOC	Super optimal broth with catabolite repression
SPR	Surface plasmon resonance
STAB1	Stabilin-1
STAT3	Signal transducer and activator of transcription 3
Syk	Spleen tyrosine kinase
Tc	Cytotoxic T
TCR	T cell receptor
TGF	Tumour growth factor
Th	T helper
TICAM1	Toll-interleukin-1 receptor domain-containing molecule 1
TIRAP	Toll-interleukin-1 receptor -associated protein
TLR	Toll-like receptor
TNF	Tumour necrosis factor
Tr1	Type 1 regulatory T
TRAM	Toll-interleukin-1 receptor domain-containing adaptor protein inducing interferon- $\beta$ -related adaptor molecule
Treg	Regulatory T
TRIF	Toll-interleukin-1 receptor domain-containing adaptor protein inducing interferon- $\beta$
TSLP	Thymic stromal lymphopoietin
VCAM-1	Vascular cell adhesion molecule-1
VIP	Vasoactive intestinal peptide
WT	Wild type

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ZAP-70      Zeta-chain-associated protein kinase 70  
cMEM      Minimum Essential Medium Eagle Alpha Modification

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## 1 Introduction

This study was set out to characterise ‘Japanin’, a DC-modulatory lipocalin only recently identified in salivary gland extract (SGE) of *Rhipicephalus appendiculatus* ticks. Japanin was found to arrest the differentiation and reprogramme the maturation of human monocyte-derived dendritic cells (moDC). Therefore, we will start by elucidating the role of DC in the immune system, including phenotypes and functions of specialised subsets, immunogenic and tolerogenic maturation, as well as interactions with T cells. Next, two classes of pattern recognition receptors (PRRs) will be introduced: Toll-like receptors (TLRs), since our studies have shown that Japanin modifies TLR-induced maturation of moDC; and C-type lectin receptors (CLRs), since we have identified CD206 / mannose receptor as a Japanin-binding receptor. In order to demonstrate how other researchers have successfully identified and characterised tick SGE-derived immunomodulatory proteins, we will review the example of Salp15. In this context, also the great biopharmaceutical potential of SGE-derived compounds will be highlighted. Moreover, relating to the structure and function of Japanin, relevant examples of immunoactive lipocalins from humans and ticks will be given. Afterwards, we will present a summary of the studies on Japanin that have so far been conducted by others. This includes the identification, cloning, and resolution of the crystal structure of Japanin. Transitioning to the experimental chapters, we will close the introduction with the aims and objectives of this study. The following chapter 2 contains a description of all materials and methods. The results are presented in chapter 3, subdivided into 4 parts. Each part of the results will be discussed at first separately in chapter 4, followed by general conclusions and perspectives for future studies.

## 1.1 Dendritic cells

Dendritic cells are part of the immune system, which is designed to protect the body from pathogenic infections or tissue damage. The importance of a fully functioning immune system is demonstrated by diseases in which it fails; either by hypofunction, which may lead to infectious disease or cancer, or hyperfunction, which may result in autoimmune disease or allergy. When the immune system malfunctions, therapeutic agents are used to support or restore appropriate responses. Immunosuppressive drugs for instance, can help prevent immunity towards innocuous antigens that were falsely classified as harmful. On the other hand, prophylactic vaccines may induce immunological memory against an antigen before the corresponding pathogen enters the body, so that the defence mechanisms are ready to act immediately in the case of an actual infection.

There are several lines of defence in the immune system. The first barrier for invading pathogens is usually represented by the epithelial surfaces as part of the skin on the outside body and mucosal tissues on the inside, for example in mouth, nose, lung, and gut. These tissues are not only physical barriers, but also contain immune cells which sense pathogens and other danger signals and activate further components of the immune system <sup>1,2</sup>. One of these early responders are DC, which fulfil a special role because they link innate to adaptive responses. DC were originally identified based on their stellate morphology as a novel type of immune cell in the spleen of mice by Ralph Steinman and Zanvil Cohn in 1973 <sup>3</sup>. Apart from their special morphology, it soon became evident that DC play pivotal roles in the immune system, distinct from the functions of macrophages, which were believed to be the key initiators of adaptive immune responses at that time <sup>4</sup>. Subsequent studies of mixed leukocyte reactions (MLRs) proved that dendritic cells are the principal and most potent stimulators of T cells <sup>5</sup>. Resident in most peripheral tissues,

DC constantly sample the environment for pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) <sup>4,6-8</sup>. DC selectively detect these conserved compounds with innate PRRs, process portions to peptides and present them as antigens to adaptive immune cells <sup>9</sup>. It is this function of DC as antigen-presenting cells (APCs) that enables specific immune responses against a particular antigen in the form of cell-mediated immunity or tolerance by T cells, or humoral release of antibodies by B cells.

### 1.1.1 DC subsets

Common DC progenitor cells in the bone marrow develop into either plasmacytoid DC (pDC) or precursors of conventional DC (pre-cDC). Both cell types enter the blood stream and exit into secondary lymphoid organs, such as the spleen and lymph nodes, or non-lymphoid tissues, such as the skin, intestine, lung, skeletal muscle, and liver <sup>10</sup>.

#### *Plasmacytoid DC*

In steady state, pDC look like plasma cells (hence their name), but they develop the DC-typical stellate morphology upon stimulation. Characteristic for human pDC is the expression of the interleukin (IL)-3 receptor / CD123, as well as BDCA-2 / CD303 and BDCA-4 / CD304, while mouse pDC characteristically express Siglec-H and BST2 / CD317 <sup>11,12</sup>. It is a functional specialisation of pDC to produce high quantities of type I interferons (INF- $\alpha$  and IFN- $\beta$ ), which activates other immune cells, such as natural killer cells, to provoke an anti-viral response <sup>13</sup>.

### *Conventional DC*

Under steady state conditions, bone marrow-derived pre-cDC constitutively migrate into lymphoid organs and differentiate into cDC under the influence of the cytokine Flt3L, while they only move into non-lymphoid tissues in response to inflammation. Conventional DC are constantly replenished by pre-cDC from the bone marrow, because their life is limited to approximately 3-5 days. Two different populations of cDC have been identified in humans and mice, independently of their tissue distribution, but based on their specialised functions and phenotype <sup>11,14</sup>:

BDCA-1 / CD1c<sup>+</sup> cDC in humans resemble CD11b<sup>+</sup> cDC in mice <sup>15</sup>. We refer to CD1c<sup>+</sup> cDC as ‘classical’ DC within this study, as they represent the major subset <sup>16</sup>. Moreover, they show all the features we classically associate with myeloid DC, such as the priming of naïve CD4<sup>+</sup> and CD8<sup>+</sup> T cells, the production of cytokines and chemokines, and the expression of a wide range of PRRs <sup>17,18</sup>.

Human BDCA-3 / CD141<sup>+</sup> cDC are often referred to as the ‘cross-presenting’ subset, even though also CD1c<sup>+</sup>, moDC, and pDC are able to load exogenous antigens on MHC class I molecules <sup>18–22</sup>. The equivalent populations of CD141<sup>+</sup> cDC in mice are CD8<sup>+</sup> DC in the spleen and CD103<sup>+</sup> DC in the intestine <sup>23,24</sup>. Both cell types share the expression of CLEC9A / DNGR-1 and more importantly XCR1, which appears to be specifically expressed by this subset in mouse and human <sup>25</sup>.

An earlier classification distinguished DC by their location in the body and refers to DC in lymphoid organs as ‘lymphoid’ or ‘resident’ DC and to those in non-lymphoid tissues as ‘interstitial’ or ‘migratory’ DC <sup>26</sup>. These populations are less well defined by phenotypic markers and overlap with the newer classification system. For example, CD14 has been

regarded as a marker for interstitial DC, while it is now associated with monocyte-derived DC, which may actually migrate less compared to other DC subsets.

### *Monocyte-derived DC*

Apart from bone marrow-derived pre-cDC, DC can also develop from CD14<sup>+</sup> blood monocytes. Monocytes may circulate in the blood stream for a few days before they migrate into tissues and differentiate into DC or macrophages<sup>27</sup>. During an inflammation, moDC can be recruited to inflamed tissues or lymph nodes to support or to replace cDC populations<sup>28</sup>. More recent studies have shown that monocytes can also enter non-inflamed tissues in steady state, such as the skin, intestine, and airways, where they may substitute cDC populations that only have a short life time<sup>29</sup>. However, monocytes are not just precursor cells<sup>30</sup>; they express PRRs and MHC class II molecules, are capable of phagocytosis, and produce cytokines<sup>31,32</sup>. Even without differentiation into DC, monocytes may survey peripheral tissues and transport antigens to lymph nodes<sup>33</sup>. In the skin, the ability of monocytes to differentiate into DC has been shown to be hindered by bacterial patterns, such as Lipopolysaccharide (LPS), a component from the cell wall of gram-negative bacteria<sup>34</sup>. This might be a mechanism to promote the generation of immediate innate immune responses in order to quickly defeat a local infection. The functional specialisation of moDC is not fully resolved at present. They share many features with cDC. Special tasks in the defence of infections, for example the production of inflammatory mediators, such as TNF- $\alpha$ , as well as roles in humoral responses to pathogens, and parts in the induction of IL-17-producing T helper (Th17) cells have been proposed<sup>10</sup>. Phenotypically, there is some overlap between moDC and cDC, such as the expression of CD11b. By contrast, the expression of CD14, CD206, and DC-SIGN / CD209 is characteristic for moDC<sup>35</sup>. Moreover, moDC do not express FLT3, since their

development depends on CCR2 rather than Flt3L. *In vitro*, the induction of moDC differentiation from blood monocytes by GM-CSF and IL-4 is very well established<sup>36-42</sup>. In order to investigate the effects of Japanin within this study, we have primarily worked with moDC, generated by this method from donated human blood of healthy donors.

### *Langerhans cells*

Before dermal DC were discovered, Langerhans cells (LCs) were regarded as the chief DC in the skin, constantly sampling antigens for presentation to T cells. A T cell-activating function has been demonstrated *in vitro*, yet could not be ascertained *in vivo*<sup>43</sup>. In contrast to the DC subsets above, LCs develop prenatal from foetal liver-derived monocytes that are recruited into the epidermis and are not found anywhere else in the body later in life<sup>44,45</sup>. LCs share this embryonic origin with most tissue macrophages.<sup>29</sup> Another difference to pDC, cDC and moDC is that LCs are long-lived and self-renew in the epidermis, but are not constantly replaced by bone marrow- or blood-derived progenitors<sup>46</sup>. More recent studies challenge the classification of LCs as a subset of DC, since they show a gene expression profile that is rather reminiscent of macrophages<sup>47</sup>.

### 1.1.2 DC maturation

Maturation has been described as a development that enables DC to process antigens for presentation on MHC molecules, migrate to secondary lymphoid organs and prime naive T cells<sup>10</sup>. (The priming of naïve T cells will be elucidated in more detail in chapter 1.1.3.) In steady state conditions, DC have been regarded as ‘immature’ since they express only low levels of MHC class II and co-stimulatory molecules, such as CD80, CD86, and CD40. The encounter with PRR agonists, however, triggers a maturation programme in the until then immature DC<sup>48,49</sup>. In this context, TLRs are associated with downstream signalling

events of antigen recognition, while CLRs are thought to play a major role in antigen uptake<sup>50</sup>. Functionally, DC maturation has also been characterised by the loss of endocytic activity and the acquisition of the ability to prime naïve T cells. Phenotypically, this may be indicated by downregulation of PRRs and upregulation of co-receptors for the stimulation or inhibition of T cell responses as well as the secretion of specific cytokines, such as IL-12<sup>51</sup>. Furthermore, mature DC can express chemokine receptors, which direct their migration from the periphery into T cell areas of secondary lymphoid organs<sup>52,53</sup>. This migration enables DC to interact with naïve T cells and thereby initiate an adaptive immune response. However, this is not always initiated by the recognition of PAMPs or DAMPs; DC constantly traffic from the periphery to secondary lymphoid organs and present antigens from apoptotic cells to T cells<sup>49</sup>. In contrast to interactions with T cells during inflammation, this steady-state migration of DC results in tolerance, rather than in immunity, against the presented antigens. Moreover, these homeostatic migratory DC do not express co-stimulatory molecules, suggesting that functionally mature DC (i.e. they are able to migrate and interact with T cells) do not necessarily show the conventional mature DC phenotype. For that reason, distinctions between ‘immunogenic’ and ‘tolerogenic’ maturation of DC have recently been introduced<sup>10,49</sup>. Immunogenic maturation is defined as in response to a PRR trigger in the form of PAMPs or DAMPs and involves the upregulation of MHC class II and co-stimulatory molecules, the expression of CCR7 and migration to T cell areas in lymph nodes, as well as the secretion of cytokines which may direct T cell differentiation into specialised effector subsets or activate other immune cells. Tolerogenic maturation on the other hand, is thought to occur constitutively under steady state conditions and also involves upregulation of MHC class II molecules and CCR7-dependent trafficking to T cell zones in lymph nodes. However, tolerogenic DC do not

express co-stimulatory molecules or secrete inflammatory cytokines, but may express co-inhibitory surface molecules and produce IL-10. They are still able to interact with naïve T cells, but rather than inducing immunity, they educate T cells to tolerate specific antigens. Thereby, tolerogenic maturation of DC represents a mechanism to eliminate autoreactive T cells that have escaped central tolerance, and to build tolerance to innocuous and commensal antigens in peripheral tissues. Some PRRs can recognise self-antigens, such as nucleic acids from dying cells, but since only few individuals develop autoimmune disease, it appears that PRR triggering is not only involved in immunogenic but also in tolerogenic maturation in healthy individuals. In fact, TLR signalling has recently been reported to be crucial for immune homeostasis and tolerance in the gut flora<sup>54</sup>. Hence there must be additional stimuli that determine whether DC undergo immunogenic or tolerogenic maturation. However, at present the exact mechanism of tolerogenic DC maturation is still unresolved.

### 1.1.3 DC - T cell interactions

All nucleated cells of the body present endogenous antigens on major histocompatibility complex (MHC) class I molecules, which when recognised as foreign or altered by a T cell receptor (TCR) on CD8<sup>+</sup> cytotoxic T cells results in killing of these cells. On the other hand, only professional APCs (including DC, macrophages, and B cells) can digest exogenous antigens for loading on MHC class II molecules to interact with CD4<sup>+</sup> T helper cells. DC are special in this respect, since they are able to prime naïve CD4<sup>+</sup> and CD8<sup>+</sup> T cells with a specific antigen, which drives T cell differentiation into effector cells<sup>55,56</sup>. Effector T cells are short-lived and die by apoptosis after exertion of their function. A small proportion of primed T cells may however differentiate into long lived memory cells,

which reside in lymphoid organs or peripheral tissues to provide rapid protection upon reencounter with the same antigen <sup>57</sup>.

### *T cell priming*

Three crucial sets of signals are provided by DC in the activation process of naïve T cells, called ‘priming’, which enables T cells to proliferate and subsequently differentiate into subsets <sup>58</sup>. The first signal includes a peptide antigen, presented on an MHC molecule, which binds its specific TCR on a T cell. At the same time CD4 or CD8 co-receptors, which associate with the TCR in a complex, bind to class II or class I MHC molecules respectively. The second signal includes the engagement of the co-stimulatory molecules B7-1 / CD80 and B7-2 / CD86 on DC with receptors on T cells. Naïve T cells express only few co-stimulatory or co-inhibitory receptors. CD28 appears to be the predominant co-stimulatory receptor for the priming of naïve T cells, although other molecules may act in place to a limited extend <sup>59</sup>. Ligation of CD28 with CD80 or CD86 enhances the production of IL-2 and thereby promotes T cell survival, proliferation, and differentiation into subsets <sup>60</sup>. The inducible T cell co-stimulator (ICOS) is another molecule on T cells that increases proliferation and function of effector cells, yet it is expressed only after priming <sup>58</sup>. The absence of co-stimulatory molecules on DC, while presenting antigens to naïve T cells, results in deletion, anergy, or the generation of regulatory T cells <sup>61,62</sup>. DC may also directly suppress T cell responses by the expression of co-inhibitory molecules, such as CD274 / PD-L1 / B7-H1. CD274 binds to PD-1 on T cells and inhibits effector T cells while promoting the generation of regulatory T cells <sup>58</sup>. In turn, regulatory T cells express co-inhibitory molecules, such as CTLA4, to suppress effector T cells by competing with CD28 and ICOS for ligation with CD80 and CD86. The binding of CTLA4 to CD80 and CD86 may mediate trans-endocytosis of the co-stimulatory molecules from the DC

surface inside T cells, thereby disabling DC to stimulate other T cells <sup>63</sup>. The third set of signals comes in form of cytokines, which determine differentiation of T cells into specialised subsets of effector cells. Signalling through ICOS may be partly involved, as it promotes the production of subset-specific cytokines, such as IL-4, IL-10, and IL-21. Further polarising cytokines may be provided by DC or other cells, as elucidated in the following specifically for each T helper cell subset <sup>64</sup>. Once CD4<sup>+</sup> T helper cells have been activated and have differentiated into effector T cells, they are able to act upon recognition of their specific antigen on another APC. CD8<sup>+</sup> cytotoxic T cells however, may need additional support by activated T helper cells, which licence DC to fully activate cytotoxic T cells, for example by expression of CD40 ligand, engaging with CD40 on DC <sup>65</sup>. Furthermore, activated CD4<sup>+</sup> T cells promote IL-2, which serves as a growth factor in the proliferation of CD8<sup>+</sup> T cells <sup>66</sup>.

In order to prime both CD4<sup>+</sup> and CD8<sup>+</sup> T cells with the same antigen, DC must possess the capacity to bring exogenous antigens into the endogenous MHC loading pathway and cross-present them on class I molecules <sup>67-71</sup>. Since the processing of exogenous antigens begins with their uptake, cross-priming DC apparently possess a unique endocytic sorting system that diverts selected exogenous antigens and allows their loading onto MHC class I molecules <sup>72</sup>. One study suggests that CD206 is essentially involved in cross-presentation by recruiting intracellular translocation factors in mouse bone marrow-derived DC <sup>73</sup>. However, another study has shown that CD206 is expendable for cross-presentation by conventional mouse DC in steady state <sup>74</sup>. Yet cross-presentation was less efficient in CD206-deficient splenic mouse moDC in the same study, this was most likely only due to impaired antigen uptake.

### *T cell polarisation*

Activated T cells can polarise into different specialised subsets of effector cells, in order to facilitate the generation of the best suited immune response. Different CD4<sup>+</sup> T helper cell subsets are characterised by their cytokine production profile and distinct functions: Th1 cells produce IFN- $\gamma$  and support responses against intracellular bacteria and viruses; Th2 cells produce IL-4 in response to infections by helminth parasites; Th17 cells produce IL-17 and are important for immunity against extracellular bacteria and fungi; and regulatory T (Treg) cells produce IL-10 or TGF- $\beta$  to control inflammation and prevent autoimmunity <sup>64</sup>. More recently, also Th9, Th22, and T follicular helper cells (Tfh) have been described, but are less well established. The expression of co-stimulatory surface molecules and the secretion of certain cytokines by DC during the priming process significantly contribute to the polarisation of T cells into effector subsets. Furthermore, it has been proposed that also the tissue in which antigens are presented influences the polarisation of T helper cells <sup>75</sup>.

### ***Polarisation of Th1 cells***

Th1 cells mediate immunity against infections with viruses, bacteria, and protozoa by producing IFN- $\gamma$ . This cytokine stimulates M1 macrophages, CD8<sup>+</sup> cytotoxic T cells, and B cells to eliminate intracellular pathogens, kill infected cells, or produce antibodies, respectively <sup>64</sup>. TLRs on the DC surface, such as TLR4, especially detect outer patterns of pathogens, while intracellular TLRs, such as TLR3, predominantly recognise nucleic acids of viruses and bacteria. Stimulation of these TLRs activates the nuclear factor- $\kappa$ B (NF- $\kappa$ B) and interferon regulatory factors (IRFs), resulting in the production of inflammatory cytokines, such as IL-12 and INF- $\gamma$ , which then drive differentiation of Th1 cells. Apart from the presence of these cytokines, also the affinity of the TCR for the presented antigen

is key to T helper cell polarisation, in the sense that strong interactions promote Th1 cells, while weak binding favours the development of Th2 cells <sup>76</sup>. Moreover, a recent study has shown that the expression of the chemokine ligand CXCL10 by DC also contributes to the differentiation of Th1 cells by binding to the CXCR3 chemokine receptor on T cells <sup>77</sup>.

### ***Polarisation of Th2 cells***

Th2 cells develop in response to helminth parasites and produce IL-4, IL-5, and IL-13 <sup>78</sup>. IL-4 promotes class-switching of antibody isotypes in B cells and also activates M2 macrophages, which are important for tissue repair. IL-4 is also the major differentiation factor for Th2 cells <sup>79</sup>. This polarisation signal is not usually provided by DC. However, DC can secrete chemokines that recruit basophiles, which are producers of IL-4 and may synergise with DC in the induction of Th2 cells <sup>80-83</sup>. Additionally, cytokines produced by epithelial cells, such as IL-33 and thymic stromal lymphopoietin (TSLP), have been associated with the polarisation of Th2 cells; and a proposed role for IL-25 is under debate <sup>84-88</sup>. TSLP has been reported to increase the expression of the co-stimulatory molecule OX40L by DC, which may facilitate the polarisation of Th2 cells, and moreover could stimulate DC to produce basophil-attracting chemokines <sup>80,86</sup>. Dendritic cells have been shown to promote the development of Th2 cells upon stimulation through certain CLRs, such as Dectin-1 / CLEC7A, or TLRs, such as TLR2, which may inhibit the production of IL-12 in a ligand-dependent manner and thereby endorse Th2 polarisation <sup>89</sup>. Furthermore, it has been demonstrated that the Notch ligand Jagged on APCs may bind to Notch 1 and Notch 2 on T helper cells, which could initiate differentiation into Th2 cells <sup>90,91</sup>. As mentioned in the context of Th1 polarisation, also the affinity of the TCR has influence on the differentiation of Th2 cells <sup>76</sup>. Independently of IL-4, weak interactions between the TCR on CD4<sup>+</sup> T cells and peptide-presenting MHC class II molecules on DC

may result in Th2 cell induction. The secreted T2 ribonuclease omega-1 from soluble egg extract of *Schistosoma mansoni* has been identified as a factor that may promote Th2 polarisation by reducing the strength of the binding between DC and T cells <sup>92</sup>. More recent studies have shown that omega-1 may moreover interfere with protein synthesis in DC and could degrade ribosomal and messenger RNA that is required for the polarisation of Th2 cells <sup>93</sup>. This pathway involved binding of omega-1 and its internalisation into DC mediated by CD206.

### ***Polarisation of Th17 cells***

Th17 cells are important for immune responses against fungi and bacteria since they secrete inflammatory cytokines, such as IL-17, IL-22, and TNF- $\alpha$ , as well as GM-CSF, which recruit innate immune cells, such as neutrophils <sup>94</sup>. On the other hand, Th17 cells have increasingly been associated with autoimmune diseases. Initially IL-23 was thought to be the key inducer for Th17 cell polarisation, but following studies identified other relevant cytokines, including IL-6, TGF- $\beta$ , and IL-1 $\beta$ , which may be provided by DC <sup>95-99</sup>. Th17-polarising cytokines can be induced in DC in a Syk-dependent manner by stimulation of CLRs, such as Mincle, Dectin-1, Dectin-2, and CD206, with fungal and mycobacterial patterns. The recruitment of the tyrosine kinase Syk activates the adaptor molecule CARD9, leading to translocation of NF- $\kappa$ B into the nucleus and transcription of IL-23, IL-6, and IL-1 genes. Co-stimulation through TLRs may enhance the production of IL-23 and IL-6. Moreover, CLRs may promote Th-17-polarising cytokines such as IL-23 by inhibiting the production of TLR-induced IL-12 <sup>100</sup>. IL-21 may promote the differentiation of Th17 cells as well, yet it was shown to be dispensable for the induction of Th17 cells in experimental myelin oligodendrocyte glycoprotein-induced autoimmune encephalitis and

autoimmune myocarditis <sup>101</sup>. Furthermore, commensal bacteria in the gut have been shown to be able to induce Th17 cell differentiation <sup>102</sup>.

### ***Polarisation of Treg cells***

Treg cells suppress immune responses in order to control an inflammatory response, maintain homeostasis or prevent autoimmunity. Therefore they secrete anti-inflammatory cytokines, such as TGF- $\beta$  or IL-10, or express co-inhibitory molecules on their surface, such as CTLA4, that may ligate with receptors on other immune cells <sup>103</sup>. Treg cells can have different phenotypes, depending on their origin. 'Natural' forkhead box P3 (FOXP3)<sup>+</sup> CD25<sup>+</sup> CD4<sup>+</sup> Treg cells originate from the thymus. During the development of natural Treg (nTreg) cells in the thymus they interact with local APCs that display self-antigens and educate nTreg cells to tolerate these antigens <sup>104</sup>. This mechanism of central tolerance is enabled by the autoimmune regulator (AIRE), a transcription factor in medullary thymic epithelial cells (mTEC), which activates genes for the expression of several antigens that are usually only expressed in specialised peripheral tissues <sup>105</sup>. These tissue-specific self-antigens can be directly presented to T cells as endogenous antigens by mTEC, or may be taken up and presented as exogenous antigens by DC. Additionally, DC may also transport antigens from the periphery into the thymus <sup>106</sup>. According to the affinity model of T cell selection in the thymus, T cells that do not bind antigens in the thymus die by neglect, while T cells that bind too strongly are deleted in order to prevent autoimmunity. Hence, selection of natural Treg cells requires an appropriate antigen binding affinity in-between.

Apart from their role in central tolerance, DC actively support homeostasis by the induction of Treg cells in the periphery <sup>107</sup>. This generally involves IL-10, TGF- $\beta$ , retinoic acid (RA), indoleamine-2,3-dioxygenase (IDO) and vitamin D<sub>3</sub>. Some DC subsets are

particularly efficient in the induction of regulatory T cells, and also the tissue or local microenvironment is important. Four different types of inducible Treg cells have been described: Type 1 regulatory T (Tr1) cells, inducible FoxP3<sup>+</sup> Treg cells, Th3 cells, and double negative (DN) (CD4<sup>-</sup> CD8<sup>-</sup>) Treg cells.

#### *Tr1 cells*

Tr1 cells can develop from naïve T cells and characteristically produce IL-10, but do not express FoxP3. Their induction has been shown to depend mainly on IL-10, provided by DC during the priming process <sup>108,109</sup>. Additional differentiation factors appear to be IL-27, TGF-β, and ICOSL. FoxP3<sup>+</sup> Treg cells may promote the secretion of these Tr1-polarising cytokines in DC <sup>110</sup>. Moreover, IFN-γ has been reported to play a role in the generation of Tr1 cells, since it inhibits Th17-polarising factors and instead induces IL-27 <sup>111,112</sup>. Furthermore, vitamin D3 was shown to lead to tolerogenic maturation of DC, resulting in increased expression of CD274 and consequently IL-10, since the binding of CD274 to PD-1 on T cells may enhance the production of IL-10 in DC <sup>113,114</sup>. Several other factors that stimulate the secretion of IL-10 by DC have also been associated with the polarisation of Tr1 cells, including vasoactive intestinal peptide (VIP), prostaglandin E2-synthesising cyclo-oxygenase-2 (COX-2), and certain peptides from pathogens <sup>115-118</sup>. The mechanism used to induce Tr1 cells might depend on the tissue they are induced in. A hepatic subset of DC may favour the development of Tr1 cells mainly by preferential expression of IL-27 <sup>119</sup>, while DC in the lung upregulate ICOSL <sup>120</sup>, splenic DC produce high levels of TGF-β <sup>121</sup>, and pDC in peripheral blood express IDO <sup>122</sup>.

### *Inducible FoxP3<sup>+</sup> Treg cells*

The expression of FoxP3 can be induced in naïve CD4<sup>+</sup> CD25<sup>-</sup> T cells in the periphery when they are activated by DC in the presence of IDO, TGF-β, vitamin D3, and RA <sup>123</sup>. IDO-expressing DC catabolise tryptophan, which may arrest the cell cycle of T cells and thereby suppress their proliferation <sup>124</sup>. Induction of Treg cells through IDO appears to be critical in DC in nasal lymph nodes <sup>125,126</sup>. A TGF-β dependency for the generation of inducible FoxP3<sup>+</sup> Treg (iTreg) cells has been shown in skin-resident LCs, in splenic DC, and in pulmonary DC <sup>127–130</sup>. Moreover, DC that have taken up apoptotic DC may produce TGF-β and induce iTreg cells <sup>131–133</sup>. In the skin, vitamin D3 has been shown to be crucial for the induction of the receptor activator of NF-κB ligand (RANKL) on keratinocytes, which stimulates DC through RANK to promote iTreg cells <sup>134–136</sup>. Moreover, CD103<sup>+</sup> DC in the gut and mesenteric lymph nodes of mice, were found to generate iTreg cells and produce high amounts of RA and TGF-β, suggesting that this is the main mechanism for iTreg polarisation upon oral antigen uptake <sup>137–141</sup>. On the other hand, iTreg cell-promoting DC in mesenteric lymph nodes additionally express COX-2 and CD274, which have also been shown to be important in this matter <sup>142,143</sup>. Furthermore, prostaglandin-D2 and VIP have been shown to drive DC along a tolerogenic maturation path and promote polarisation of iTreg cells <sup>144–146</sup>.

### *Th3 cells*

Th3 cells typically secrete high amounts of TGF-β but only little IL-10 and IL-4. Th3-derived TGF-β is sufficient to induce FoxP3 expression in activated T cells and may also promote FoxP3 in Th3 cells <sup>147,148</sup>. The polarisation into Th3 cells has been shown to be inducible by oral administration of antigens, such as myelin basic protein <sup>149</sup>.

### *DN Treg cells*

DN Treg cells, expressing neither CD4 nor CD8, have been shown to produce IFN- $\gamma$  and kill DC and effector T cells by the induction of apoptosis in a Fas/Fas ligand-dependent manner, and may also kill B cells and NK cells in a perforin/granzyme-dependent fashion<sup>150–152</sup>. Moreover, DN Treg cells may downregulate the expression of co-stimulatory molecules on DC by interaction with CTLA4, and additionally secrete IL-10. DC have been utilised for *ex vivo* expansion of DN Treg cells, yet their mode of activation is not fully understood at present.

## 1.2 Pattern recognition receptors

Pattern recognition is the first step in the development of an immune response. DC mature in response to this stimulation, including the processing and presentation of antigens to T cells and the secretion of cytokines to activate or inhibit other immune entities. This function places DC at the interface between innate and adaptive immunity. Highly selective PRRs are expressed on the DC surface, inside specialised intracellular compartments, or the cytosol. These include TLRs, CLRs, nucleotide-binding oligomerisation domain (NOD)-like receptors (NLRs), and retinoic acid-inducible gene I (RIG-I)-like receptors. Each PRR is selective for certain molecules, such as proteins, carbohydrates, or nucleic acids. The detection of conserved PAMPs or DAMPs then triggers signalling cascades inside DC that result in the activation or inhibition of specific genes.

### 1.2.1 Toll-like receptors

TLRs are an important and probably the best characterised group of PRRs expressed by professional APCs. They are able to identify a wide range of patterns and initiate the up-

regulation of co-stimulatory molecules for T cell activation as well as the production of inflammatory cytokines by activation of transcription factors such as NF- $\kappa$ B<sup>153,154</sup>.

### *Selective TLR expression by different DC subsets*

Ten TLRs (TLR1-10) have been described in humans to date, with TLR1-9 having conserved functions in human and mice, while TLR10 is considered non-functional in mice<sup>155</sup>. Mice additionally express TLR11-13, which are not found in humans. More TLRs have been described in other vertebrate species, such as fish<sup>156</sup>. In humans and mice, the expression pattern of TLRs appears to be specific for certain DC subsets<sup>10</sup>. Plasmacytoid DC of both species express TLR7 and TLR9, which recognise single-stranded RNA and unmethylated CpG DNA, common motifs of bacteria and viruses. During viral or fungal infections, XCR1<sup>+</sup> DC are thought to cross-prime cytotoxic T cells for killing of infected cells and therefore express high levels of TLR3, which detects double-stranded RNA<sup>157,158</sup>. On the other hand, the expression of TLR3 on DC in the gut has recently been reported to uphold immune homeostasis. These distinct outcomes upon triggering the same TLR suggest that the stimulus, the environment, or the type of DC, rather than the TLR, may decide whether DC mature in an immunogenic or tolerogenic manner<sup>159</sup>. While the TLR expression profiles of pDC and XCR1<sup>+</sup> DC seem to focus on patterns of intracellular pathogens or endocytosed self-antigens, classical DC (CD1c<sup>+</sup> in humans or CD11b<sup>+</sup> in mice) and moDC appear specifically equipped for extracellular stimuli as well as intracellular products of bacteria and viruses<sup>10</sup>. Classical DC and moDC of human and mouse typically express high levels of TLR1, 2, 4, 5 and 6. The subset-specific expression of other TLRs is not conserved between human and mouse. For example, TLR8 is expressed by all DC subsets in humans except pDC, whereas in mice TLR8 is only found on monocytes and to a little degree on moDC. Moreover, TLR9 is exclusively present on pDC in humans, but

expressed by all DC subsets in mice. These differences in the TLR expression profile suggest that similar DC subsets in human and mouse may process some stimuli differently.

### *TLR signalling*

The binding of TLR ligands induces conformational changes in TLRs that lead to the recruitment of intracellular adaptor molecules. Four different adaptor molecules have been identified in this context: MyD88, MAL / TIRAP, TRIF / TICAM1, and TRAM <sup>160</sup>. Selective recruitment of these molecules may partly explain how different TLR ligands induce distinct responses. For pro-inflammatory cytokine production, MyD88 is the key adaptor molecule of all TLRs except TLR3, which utilises the adaptor molecule TRIF to induce type I interferons. Recruitment of MyD88 triggers signalling cascades involving mitogen-activated protein kinases (MAPKs) and activation of NF- $\kappa$ B and other transcription factors, mediating the expression of pro-inflammatory cytokines, such as IL-6, IL-12, and TNF- $\alpha$ . TLR4 may also signal via TRIF, resulting in the activation of IRFs and the production of type I interferon. Moreover, maximal induction of co-stimulatory molecules, such as CD86, via TLR4 has been shown to depend on synergistic signalling via MyD88 and TRIF, enhancing the transcription of genes that encode co-stimulatory molecules <sup>161–163</sup>. Interestingly, LPS-induced activation of TLR4 depends less on TRIF in DC than in macrophages, showing that these cell types can signal differently through the same receptor <sup>163–165</sup>. Plasmacytoid DC efficiently activate IRFs through MyD88 signalling upon TLR7 and TLR9 stimulation. This might be a functional specialisation of pDC and may explain why they produce such high quantities of type I interferon compared to other DC subsets. Consequently, further functional specialisations of different DC subsets might

also be a result of selective utilisation of signalling molecules in TLR pathways, at least in part.

Certain TLRs have soluble or membrane-bound co-receptors <sup>166</sup>. TLR4 for example detects LPS after its capture by the LPS-binding protein (LBP) in the serum and subsequent binding of the complex to the co-receptors CD14 and MD-2 in the plasma membrane <sup>167,168</sup>. While CD14 is only responsible for the binding of LPS and lacks an intracellular signalling domain, TLR4 and its accessory protein MD-2 can transduce signals inside the cell.

### 1.2.2 C-type lectin receptors

CLRs are a class of PRRs named after the first identified members of this group, which predominantly bind carbohydrate-containing ligands in a calcium-dependent manner. Apart from this activity that is mediated by their lectin-like domains, CLRs may also bind non-glycosylated proteins, lipids, or inorganic molecules, depending on the overall molecular structure of the receptor <sup>169</sup>.

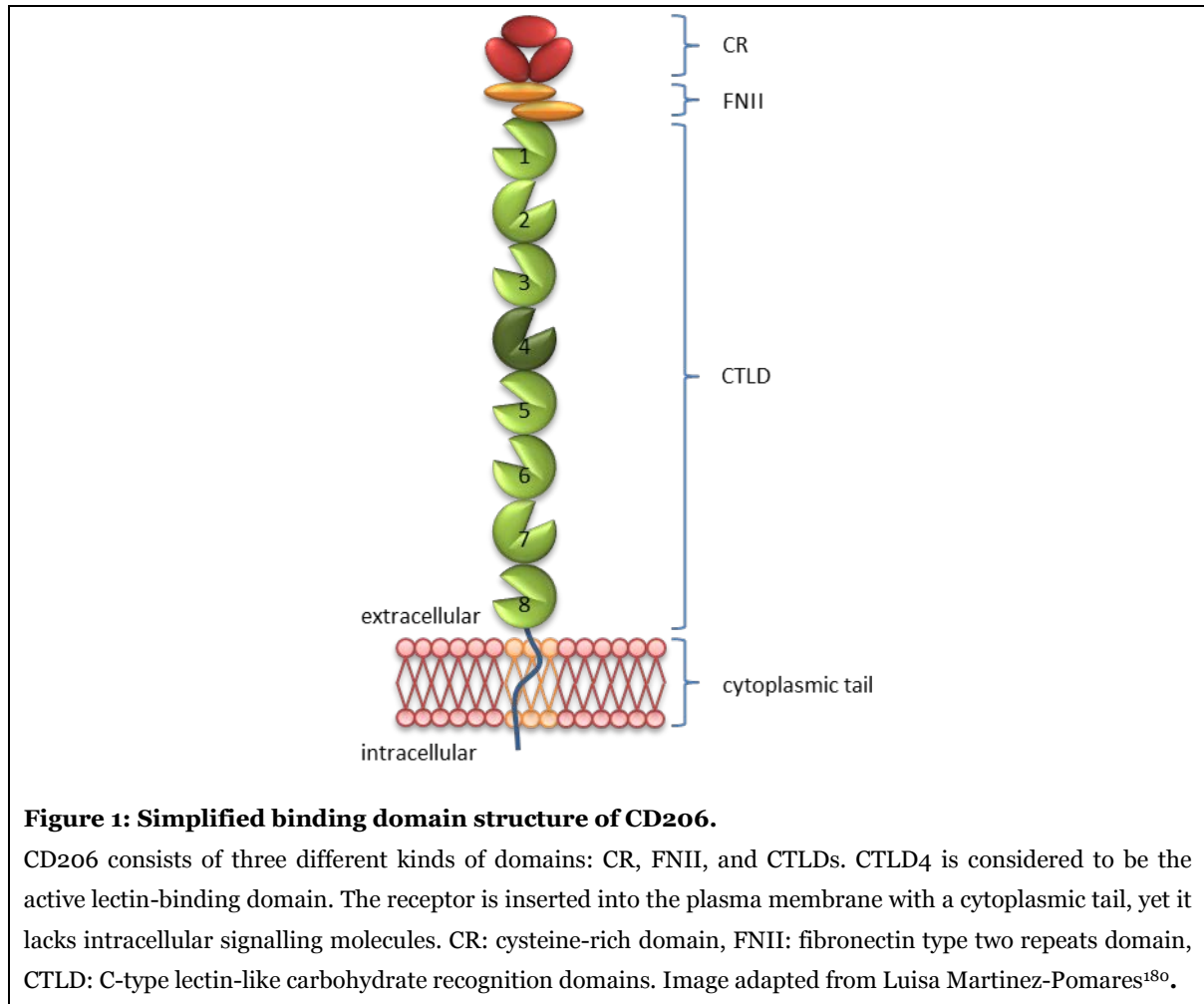
#### *Signalling through CLRs*

CLRs are often associated with phagocytosis and subsequent antigen processing, but ligand recognition can also directly mediate activation of specific genes, for instance resulting in the secretion of cytokines. One example of a signalling CLR is Dectin-1. Stimulation of Dectin-1 with zymosan, a glucan from the cell wall of yeast, has been shown to recruit Syk, initiating further down-stream signalling events which independently of MyD88 resulted in translocation of NF- $\kappa$ B into the nucleus and subsequent IL-2 and IL-10 gene transcription <sup>170</sup>.

Then there are CLR s that cannot induce the translocation of NF- $\kappa$ B directly, but modulate the activated transcription factor after it has been induced to translocate by signalling molecules from TLR pathways. DC-SIGN for example alters the outcome of TLR4 stimulation when both receptors are triggered simultaneously, as by *Mycobacterium tuberculosis*<sup>171–174</sup>. Without this PRR ‘cross-talk’, TLR4-activated NF- $\kappa$ B would promote the production of pro-inflammatory cytokines. But the stimulation through DC-SIGN at the same time triggers the activation of the serine and threonine kinase Raf-1, inducing acetylation of the NF- $\kappa$ B subunit p65, which promotes anti-inflammatory cytokine responses by mediating enhanced and sustained IL-10 gene transcription. Similar mechanisms of immune modulation have been described for yeast (e.g. *Candida albicans* and *Saccharomyces cerevisiae*) and viruses (e.g. HIV-1 and measles virus)<sup>175</sup>.

### *CD206*

CD206 is a CLR found on several DC subsets, including human moDC, inflammatory mouse bone marrow-derived DC (BMDC), as well as macrophages and specialised epithelial cells of both species<sup>176–179</sup>. As a recycling receptor, CD206 constantly traffics between the plasma membrane and early endosomes, with about 70 % of the receptors located intracellularly in steady state. CD206 mediates clathrin-dependent endocytosis of a variety of ligands, which are typically mannose-terminated polysaccharides or glyco-conjugates<sup>180</sup>. The 175 kDa membrane protein consists of a cysteine-rich (CR) head domain, a fibronectin type II domain (FNII), multiple C-type lectin-like domains (CTLDs), and a cytoplasmic tail (Figure 1).



Each domain can bind different types of ligands <sup>180,181</sup>: the CR domain binds sulfated carbohydrates; the FNII domain binds collagens; and the CTLDs – in particular CTLD4 – bind mannose-, fucose-, or N-acetyl-D-glucosamine-terminated glycans in a calcium-dependent manner <sup>182–184</sup>. Like many CLRs, CD206 lacks transmembrane signalling motifs, yet it is thought to be involved in modulation of the signalling pathways of other PRRs <sup>178,185–187</sup>. Recently, a role for CD206 in the recruitment of intracellular translocation factors has been proposed in the context of cross-presentation, yet other studies suggest it might be dispensable <sup>73,74</sup>. Moreover, an involvement in immune homeostasis and surveillance has been suggested, as based on associations between genetic variation of the CD206 gene and autoimmune diseases <sup>179,188,189</sup>. Furthermore, studies with CD206-

deficient mice suggest that CD206 is crucial for clearance of glycoproteins in the serum, while it does not appear to play a role in resistance against systemic *Candida albicans* infection<sup>190,191</sup>. On the other hand, it has been reported that the CTLD4-7 domains of CD206 mediate the uptake of diverse allergens by DC. CD206 might moreover be involved in polarisation of Th2 cells, since CD206 deficiency enhanced the expression of Th1-polarising IFN- $\gamma$ , whilst it decreased the expression of Th2-polarising cytokines<sup>192</sup>. Interestingly, the same study found increased activity of IDO in CD206-deficient DC, suggesting that CD206 may down-regulate the activity of IDO, a mechanism that might contribute to allergic reactions. Overall, the outcome of an immune response upon CD206-mediated antigen uptake by DC appears to depend on environmental factors, or the presence or absence of co-stimulation through additional receptors.

### 1.3 Tick-derived immunomodulators: the example of Salp15

Ticks are blood-feeding ectoparasitic arthropods distributed all over the world, which attach to and feed on mammals, birds, and some reptiles. Three families of ticks have been described to date: Ixodidae (hard ticks) comprise almost 700 species; Argasidae (soft ticks) count almost 200 species, and Nuttalliellidae consists of only one species<sup>193,194</sup>. The family Ixodidae has two subgroups, Prostriata and Metastrata, defined by the presence or absence of an elongated mouthpart, respectively. Argasid ticks are typically found on birds and feed on multiple hosts during one life cycle, with each blood meal lasting no longer than about an hour. Ixodid ticks on the other hand, may require more than a week on one host to complete feeding. Therefore, in addition to innate immune factors, hard ticks are potentially confronted with rejection by an adaptive response in the host. Yet they are able to withstand or evade this threat, possibly because of immunomodulatory mediators in their saliva, which are secreted into the host when specialised tick mouthparts penetrate

into the skin <sup>195</sup>. The active compounds identified in SGEs of different tick species to date, target multiple innate and adaptive components, such as complement factors, defensins, prostaglandins, cytokines, B cells, and T cells <sup>196</sup>. Some salivary gland products even interfere with wound healing growth factors and inhibit the coagulation system of the host, potentially contributing to the ability of hard ticks to feed on just one site for so long <sup>197–199</sup>. A number of studies suggest that smaller pathogens have co-evolved with ticks and use them as transmission vectors inside their host, thereby evading elimination by exploitation of the saliva-mediated manipulation of the host immune system <sup>200–206</sup>. Hence, targeting immunomodulators in tick saliva might provide a strategy to combat the transmission of tick-borne pathogens <sup>207</sup>. On the other hand, tick SGEs potentially contain an array of compounds that could be reproduced in the lab and used for therapeutic immunosuppressive applications.

To date, Salp15 is one of the best characterised tick-derived immunomodulators <sup>208</sup>. The 15 kDa protein has been identified in saliva of the prostriate tick species *Ixodes scapularis* by Anguita and colleagues and was shown to inhibit the activation of naïve T cells by binding to CD4 <sup>209</sup>. Additionally, it has been demonstrated that Salp15 alters the maturation of DC by binding to DC-SIGN <sup>210</sup>. More recently, also specific inhibition of keratinocyte-mediated inflammation in the skin has been reported <sup>211</sup>. While earlier studies of Salp15 have been mostly carried out with mice, more recent studies also report immunomodulatory effects of a Salp15-like protein on cytokine production by human peripheral blood mononuclear cells (PBMC) <sup>212</sup>. The homologue inhibited LPS-induced secretion of IL-10 in PBMC, but did not affect TNF- $\alpha$  or IL-6. Unravelling the mode of action of Salp15 has helped understand how ticks may withstand rejection by the host immune system during feeding. Notably, the specific binding of Salp15 to the CD4 co-

receptor on T cells is the first described for an arthropod protein, and the binding to DC-SIGN on DC revealed an as-yet-undiscovered modulation pathway of TLR2 and TLR4 signalling via MEK. Potentially, Salp15, or the mechanism by which it modulates the human immune response, could turn out useful for the prevention of pathogen transmission by ticks, the treatment of T cell-mediated allergies, or advances in HIV research; yet there are no studies that demonstrate efficacy in autoimmune diseases. In order to emphasise the potential of SGE-derived immunomodulators for medical applications, as well as to establish a niche for the unique functions of Japanin, the example of Salp15 will be reviewed in more detail in the following.

### **1.3.1 Identification of Salp15 as a T helper cell inhibitor**

Despite the generally poor immunogenicity of tick salivary proteins, antibodies against recombinant Salp15 could experimentally be raised in tick-immune rabbits and purified from their sera. These antibodies enabled the detection of Salp15 in skin biopsies of mice shortly after a tick bite, which suggested that the protein was released by the tick during feeding into the host skin. T cell activation assays, in which stimulation by APCs was mimicked by bead-coupled anti-CD3 and anti-CD28 antibodies <sup>213</sup>, demonstrated that Salp15 directly impairs proliferation, IL-2 production, and IL-2 receptor  $\alpha$  chain / CD25 expression of purified naïve T helper cells <sup>209</sup>. All these functions are associated with signalling cascades triggered by stimulation of the TCR complex. This stimulation usually triggers an intracellular signalling cascade, resulting in a calcium ion ( $\text{Ca}^{2+}$ ) influx from the ER into the cytosol <sup>214</sup>. This induces  $\text{Ca}^{2+}$  channels in the plasma membrane to open and leads to further calcium influx. The elevated levels of  $\text{Ca}^{2+}$  inside the cell then activate specific enzymes and transcription factors, such as NFAT, NF- $\kappa$ B and CREB, which drive

T cell proliferation and effector functions. A reduced  $\text{Ca}^{2+}$  mobilisation was found when TCR stimulation occurred in the presence of Salp15, potentially explaining the inhibited T cell function in part.

Subsequent studies with fluorescently labelled protein demonstrated the capacity of Salp15 to bind specifically to T helper cells <sup>215</sup>. Since pre-treatment of T cells with trypsin abolished the binding of Salp15, a protein receptor for Salp15 was suspected on the cell surface. Based on the TCR-inhibitory functions of Salp15, key components of the TCR complex were suspected as potential binding partners on T cells. Co-precipitation and confocal microscopy analysis of labelled Salp15 and receptor candidates suggested CD4 as a binding partner for Salp15, while no binding to chains of the TCR, CD3, or CD28 could be detected. The specificity of the binding of Salp15 to CD4 was confirmed in the same study by binding experiments with CD4-transfected cell lines that could be stained with labelled Salp15, whilst non-transfected cell lines and CD8<sup>+</sup> T cells could not. Moreover, pre-incubation of CD4-expressing cells with unlabelled Salp15 or agonistic anti-CD4 antibodies prevented the binding of labelled Salp15. The affinity of the binding to CD4 was determined at a  $K_D$  of 47 nM in a microtitre assay with graded doses of immobilised purified soluble CD4 and his-tagged Salp15 plus a peroxidase-conjugated anti-his tag antibody and a suitable substrate. The size exclusion chromatography profile of his-tagged Salp15 demonstrated primarily monomers and dimers in the preparations. Analysis of Salp15-CD4 complexes suggested a 1:1 molar ratio in the binding.

Ligation of a peptide-MHC complex with the TCR complex can drive T cell proliferation and induce effector functions. These events are mediated by signalling cascades, including the activation of protein kinases of the Src family <sup>216–219</sup>. Src family kinases trigger tyrosine phosphorylation of several proteins which are recruited from the cell membrane and

cytosol to the immunological synapse, where the signals are amplified and may lead to the mobilisation of  $\text{Ca}^{2+}$  from intracellular stores and the production of IL-2<sup>220–223</sup>. The repression of  $\text{Ca}^{2+}$  fluxes and IL-2 production by Salp15 was found to be due to inhibition of the Src kinase Lck and its downstream effector protein Zap-70, preventing phosphorylation of PLC $\gamma$ 1<sup>215</sup>. Moreover, the inhibition of Lck prevented the phosphorylation of LAT and Vav1, which affected actin polymerisation and thereby the formation of lipid rafts and associated amplification of signals<sup>224</sup>. Experiments with CD4-negative T cells confirmed that the activity of Salp15 was mediated by CD4.

Moreover, one study induced Salp15 in a mouse macrophage cell line and showed that the expression of Salp15 by macrophages impaired their capacity to activate CD4<sup>+</sup> T cells<sup>225</sup>. The same study utilised attenuated *Salmonella enterica* as an *in vivo* delivery vector for Salp15, which is taken up by phagocytosis only, and therefore specifically by APCs. CD4<sup>+</sup> T cells isolated from mice which had been given the Salp15 gene via the *S. enterica* delivery system, did not respond to *ex vivo* stimulation with a specific antigen. These results suggest that the induction of Salp15 expression in APCs might provide an effective approach to inhibit T cell priming *in vivo*. However, the manner in which Salp15 was delivered in this study also resulted in higher bacterial burdens, leading to splenomegaly and delayed clearance of the infection. Hence, a different way for the delivery of Sap15 into APCs would be needed to make the method applicable for medical treatments in humans.

### 1.3.2 Salp15 Homologues

Hovius and colleagues investigated whether Salp15-related proteins are produced by other *Ixodes* species<sup>226</sup>. Salp15 and three homolog genes were identified by reverse transcriptase polymerase chain reaction (RT-PCR) in *Ixodes ricinus* ticks. While one of the amino acid

sequences indicated 80 % similarity with Salp15 from *I. scapularis* evenly spread over the whole peptide, the other two homologues matched to only about 60 % and mainly aligned at the C-terminus. The DNA and protein sequences were compared to databases where more proteins with similar sequences like Salp15 from other *Ixodes* ticks were found. Later studies characterised further homologues of Salp15 in *I. persulcatus*, *I. pacific*, and *I. sinensis*; again with the highest degree of similarity at the C-terminal end, the domain that binds to the CD4 co-receptor on T-cells <sup>227-229</sup>. The findings suggested a multigene family of Salp15-like proteins conserved within the Ixodidae. Interestingly, all identified species have been associated with infections by *Borrelia burgdorferi sensu lato* bacteria. This finding suggests that Salp15 proteins may play a key role in the transmission of *B. burgdorferi sensu lato*, potentially by inhibiting adaptive immune responses in the host <sup>226</sup>.

### 1.3.3 Prevention of HIV entry by blocking the ligation of gp120 and CD4

After the identification of CD4 as the receptor for Salp15 on T cells, *ab initio* modelling of the binding demonstrated that Salp15 ligated with the D1 domain of CD4, the same domain that is used for docking by the HIV envelope protein gp120 <sup>230</sup>. Yet, the conformational changes of the receptor that occurred upon binding of one of the two ligands were found to be very different. Investigations into whether Salp15 could prevent viral entry into the cell determined P11 as the C-terminal sequence of Salp15 that mediated ligation with CD4 <sup>231</sup>. However, increasing concentrations of P11 did not cause dissociation of CD4 and labelled gp120 in a microtitre assay. On the other hand, the complete Salp15 protein was shown to reduce the fusion of derivate HeLa cell lines which expressed either gp120 or CD4, suggesting that Salp15 could block the interaction of gp120 with CD4.

Therefore, it was hypothesised that the inhibition of the binding of gp120 to CD4 by Salp15 was a result of steric hindrance due to the differential conformational changes induced by the binding of the whole Salp15 protein to the receptor. On the contrary, simple occupation of the Salp15 binding site on CD4 through P11 could not mediate these conformational changes. Furthermore, it was shown that Salp15 and P11 bound to gp120, suggesting that the inhibition of the binding of gp120 to CD4 might be due to direct interaction of Salp15 with the virus envelope protein, at least in part. Identification of the Salp15-binding residues in gp120 might prove useful, as for the generation of HIV-neutralising antibodies in order to prevent viral entry into host cells.

#### **1.3.4 Modulation of TLR signalling by binding to DC-SIGN**

In addition to the inhibitory activity on T cells, Salp15 was shown to suppress functions of human DC, as measured upon stimulation with TLR2 and TLR4 agonists by decreased production of the pro-inflammatory cytokines IL-6, IL-12, and TNF- $\alpha$  as well as limited capacity to activate T cells <sup>210</sup>. The same activity of Salp15 was observed after stimulation of DC with *B. burgdorferi*, a tick-transmitted bacterium which is detected by TLR2 <sup>210,232</sup>. The activity of Salp15 on DC was demonstrated to be due to interactions between Salp15 and DC-SIGN leading to phosphorylation of Raf-1. Interestingly, Raf-1 activated MEK, which resulted in enhanced degradation of IL-6 and TNF- $\alpha$  mRNA. Moreover, this mechanism reduced chromatin remodelling at the IL-12p35 promoter, impairing the production of IL-12. In contrast, modulation of TLR4 signalling via DC-SIGN by other pathogens, such as mycobacteria, yeast, or viruses, induced IL-10 by Raf-1-mediated acetylation of an NF- $\kappa$ B subunit (see 1.2.2). Therefore, the Salp15-triggered DC-SIGN

signalling events have been reported to be a novel TLR-modulating pathway, which acts on the transcriptional and post-transcriptional level of cytokines at the same time.

### 1.3.5 Salp15 as a target for vaccines against *Borrelia burgdorferi* infection

Since the Salp15-induced modulation of TLR signalling via DC-SIGN may occur upon co-stimulation with *B. burgdorferi* as a TLR2 agonist, *I. scapularis* ticks and *B. burgdorferi* might have co-evolved this mechanism in order to evade DC-initiated immune responses<sup>233</sup>. Hence the molecular mechanisms of immune modulation by tick salivary compounds have been considered for developing innovative therapeutic agents against tick-borne diseases, such as Lyme disease, an infection caused by *B. burgdorferi* that can result in severe organ damage<sup>234</sup>. Some individuals may develop antibodies against the bacterium, but these cannot confer immunity for the future. The development of vaccines specifically targeting *B. burgdorferi* antigens is challenging due to heterogeneity of different strains<sup>235</sup>. Hence, the current recommended prevention of Lyme disease is simply the avoidance of tick bites. A possible resolution for the future could lie in targeting immunosuppressive components in tick saliva, which might be required for the transmission of *B. burgdorferi*<sup>236</sup>. It has been suggested that *B. burgdorferi* might actively enhance the expression of Salp15 in *I. scapularis* in order to facilitate its own transmission, as indicated by increased levels of Salp15 in ticks carrying the bacterium compared to uninfected ticks<sup>237</sup>. Moreover, Salp15 and its homologue protein Iric-1 were shown to prevent antibody- and complement-mediated killing of *B. burgdorferi* by specific binding to the outer surface protein C (OspC)<sup>237-239</sup>. Consequently, more severe disease symptoms were observed in animals that had been pre-treated with Iric-1 and re-challenged with *B. burgdorferi* than in animals that had not received Iric-1. On the other hand, significantly decreased

transmission of *B. burgdorferi*, or limited capacity to infect, was observed upon siRNA-mediated knock-down of Salp15 expression in *I. scapularis* ticks<sup>237</sup>. It has been hypothesised that Salp15 might represent a receptor for OspC in ticks that mediates the entry of *B. burgdorferi* inside the salivary glands, providing an alternative explanation for the inhibited transmission of *B. burgdorferi* upon siRNA knock-down of Salp15 in ticks<sup>240</sup>. Either way, these results suggest that targeting Salp15 may represent a potential strategy for preventing Lyme disease.

Preventative vaccination with Salp15 as an antigen might generate Salp15-specific antibodies, which may mediate clearance of Salp15-coated *B. burgdorferi* by phagocytes, or might even reduce transmission of *B. burgdorferi*, if the antibodies were ingested by ticks during feeding<sup>241</sup>. In active and passive immunisation studies with Salp15 in mice, it appeared that both vaccine strategies generated immunity against *B. burgdorferi*; interestingly, whilst the feeding of ticks was not affected<sup>242</sup>. Increased efficacies were observed when *B. burgdorferi* antigens and Salp15 protein or Salp15 antiserum were administered together, suggesting that this promoted opsonisation and degradation of Salp15-*B. burgdorferi* complexes.

A recent study attempted to immunise mice, not with Salp15 protein, but a replication-incompetent adenovirus as a vector to induce the expression of Salp15 *in vivo*<sup>243</sup>. However, the delivery of Salp15 did not significantly reduce the tick-borne *B. burgdorferi* load in the host when compared to the controls. On the contrary, markedly decreased levels of IL-2 in Salp15-vaccinated animals indicated suppressed T cell responses and hence suggested that the delivery of Salp15 resulted in functional expression of the protein by host cells, rather than in immunity against it. This shows that the use of a truncated version of Salp15, without immunosuppressive properties, would represent a much safer

vaccine antigen. It has been claimed that a C-terminus-truncated mutant of Salp15 could also generate Salp15-specific antibodies, yet these data have not been published <sup>242</sup>.

### 1.3.6 Protection from allergic asthma in a mouse model

Salp15 has been reported to inhibit the development of disease in a mouse model of allergic asthma <sup>244</sup>. This experimental asthma model shares many similarities with allergic asthma in humans, which is predominantly mediated by CD4<sup>+</sup> T cells <sup>245</sup>. Since Salp15 was shown to inhibit proliferation and IL-2 production by CD4<sup>+</sup> T cells, it has been proposed that the tick salivary protein could prevent this respiratory disease. Mice were therefore sensitised and challenged with OVA-aerosols as a model for an allergic airway antigen, with and without co-administration of Salp15. The numbers of inflammatory immune cells and Th2-specific cytokines in the bronchoalveolar lavage fluid, as well as mucus production in the lungs, were reduced when the animals were co-treated with Salp15. Furthermore, Salp15 treatment prevented hyper-responsive OVA-specific IgG1 and IgE production. These findings may suggest that the administration of Salp15 might be a strategy to treat severe forms of allergic asthma in humans, while approaches to target CD4 with therapeutic antibodies have so far failed in clinical trials <sup>246-249</sup>.

### 1.3.7 Salp15 promotes autoimmunity by inducing Th17 cells in an EAE model

Limitations for the therapeutic use of Salp15 have become visible in an autoimmune disease model. Salp15 actually did not improve, but worsen, disease in mice with experimental autoimmune encephalitis (EAE) <sup>99</sup>. This mouse model relates to multiple sclerosis in humans and is mediated by myelin-specific CD4<sup>+</sup> T cells that infiltrate the central nervous system. Administration of Salp15 promoted IL-17-producing CD4<sup>+</sup> Th17

cells during the pathogenesis of EAE, leading to more severe symptoms than in control mice. Meanwhile the permeability of the blood-brain barrier was not affected by Salp15, nor were the levels of antibodies or IFN- $\gamma$ , suggesting that B cell and Th1 functions were not impaired. Salp15 enhanced the differentiation of myelin-specific Th17 cells not only *in vivo*, but also *in vitro*. In mice, the differentiation of Th17 from naïve T cells has been reported to depend on TGF- $\beta$  and IL-6<sup>95-97</sup>. Interestingly, in the presence of Salp15, TGF- $\beta$  was negligible, while IL-6 was sufficient for the induction of Th17 cells<sup>99</sup>. Since Salp15 is known to inhibit the production of IL-2, these results support a suggested role for TGF- $\beta$  in the polarisation of Th17 cells, which is the suppression of IL-2, as IL-2 may hinder the polarisation of Th17<sup>250-252</sup>.

## 1.4 Immunoactive lipocalins

Lipocalins are typically small secretory proteins of about 20 kDa with conserved cysteine residues and a barrel-formed crystal structure with an internal binding pocket for small hydrophobic ligands<sup>253</sup>. They may be glycosylated and can occur as monomers or in complexes of two or more molecules. A variety of biological and immunological functions are mediated by lipocalins, such as the transport or neutralisation of specific molecules, enzymatic functions, and the binding to specific receptors on certain cells<sup>254</sup>.

### 1.4.1 Lipocalins in the human immune system

Immunoactive lipocalins, so-called ‘immunocalins’, have been identified in a cluster of genes encoding in the q32-34 region of chromosome 9 in humans<sup>255</sup>. These lipocalins have been shown to have anti-inflammatory or anti-microbial functions, acting on T cells, B cells, NK cells, neutrophils, macrophages, monocytes, and complement. However, the exact mode of action is not yet fully understood for most of them. Some lipocalins may act

as enzymes, for instance Lipocalin-type prostaglandin (PG) D synthase (L-PGDS), which has been mainly studied in brain cells, was shown to catalyse the production of PGD<sub>2</sub> <sup>256</sup>.

A better-studied example is retinol-binding protein (RBP), which transports retinol (vitamin A) from the liver through the blood to specific tissues where it is needed, such as the eyes or epithelial tissues. Increased susceptibility to infections as a consequence of vitamin A deficiency has shown that retinol is also a crucial factor for the maintenance of an intact immune system. This might be partly due to a crucial role for retinol in the differentiation of dendritic cells as well as in the induction of tolerance in the gut-associated lymphoid tissues (GALT) <sup>257</sup>. Conventional DC in the GALT express enzymes to metabolise retinol into RA. This metabolite induces the expression of gut-homing receptors on effector T cells and IgA-secreting B cells. There is also indication that RA plays a role in the balance of the induction of Treg and Th17 cells in the GALT. Moreover, retinol has recently been shown to be essential for the differentiation of pre-DC into conventional DC in the GALT and the spleen, though the exact mechanism is not yet fully understood <sup>258–261</sup>. Besides these functions in the GALT, retinoids are also important regulators of brain activities. Since transporter molecules such as RBP would not be able to pass the blood-brain barrier, retinoids might need to change the transporter at this point. It has been suggested that L-PGDS may transport retinoids in the brain, as it was shown to bind RA <sup>262</sup>. This activity would represent an additional or alternative function to the synthesis of PGD<sub>2</sub>, making L-PGDS a bifunctional lipocalin.

Another human lipocalin that has been widely studied is glycodelin / placental protein 14 (PP14) / progesterone-associated endometrial protein, a glycosylated 28 kDa lipocalin highly expressed during early pregnancy in the reproductive tract. It was found to bind in a lectin-like manner to the leucocyte common antigen (LCA) / tyrosine phosphatase

receptor CD45 and other glycosylated compounds on the surface of T cells, leading to anti-inflammatory responses, specifically the inhibition of T cells <sup>263</sup>. It appears glycodelin arrests effector T cells in an activated state and suppresses the secretion of cytokines, but promotes the generation of Treg cells <sup>264</sup>. Additionally, it has been demonstrated that glycodelin drives tolerogenic DC maturation, as indicated by down-regulation of co-stimulatory molecules and up-regulation of IL-10 <sup>265</sup>. More recently, glycodelin has been shown to bind to L-selectin on human monocytes and macrophages in a glycan-dependent manner <sup>266</sup>. The binding of glycodelin by monocytes and macrophages resulted in increased production of IL-6, which suppressed the expression of IFN- $\gamma$  in T helper cells.

#### 1.4.2 Immunomodulatory lipocalins identified in tick SGE

Numerous tick-derived lipocalins have been reported to date. Selected relevant examples from different tick families will be given in the following.

##### *OmCI from the soft tick *Ornithodoros moubata**

Nunn and colleagues have reported the identification of the first SGE-derived lipocalin that inhibits a complement factor <sup>267</sup>. The 16 kDa protein was termed ‘OmCI’, because it is an *Ornithodoros moubata*-derived complement inhibitor.

The complement system is an innate part of the immune system, comprising a number of proteins that occur in the blood, which cause a series of reactions to facilitate the elimination of pathogens. These humoral reactions come into place very early after an infection, before the generation of adaptive responses. Three routes of complement system activation have been described: The classical, the mannose-binding lectin (MBL), and the alternative pathway. The first two pathways are initiated by the binding of antibodies or

carbohydrates to the surface of pathogens, while the latter begins after spontaneous breakage of complement factors in the blood. All three pathways eventually come to the cleavage of the complement factor C3 into C3a and C3b. The C3b fragment then forms a C5 convertase, which splits C5 into C5a and C5b. The latter instructs the formation of a membrane attack complex (MAC), leading to lysis of pathogens by forming a pore that permeabilises their membrane <sup>268</sup>. C5a on the other hand, is a very potent anaphylatoxin which directly accelerates phagocytosis of pathogens by neutrophils and monocytes <sup>269</sup>. Moreover, C5a works in combination with C3a to initiate the activation of mast cells, the recruitment of antibodies, or other complement factors, and phagocytes. These reactions are meant to defeat pathogens, but may also lead to sepsis <sup>270,271</sup>.

Because the complement can be activated so quickly, it could potentially target soft ticks, such as *O. moubata*, and mediate their elimination, although their feeding takes only minutes or hours. This might be why their saliva contains complement inhibitors, as they might enable soft ticks to complete their blood meal. OmCI represents the first natural inhibitor specifically targeting the activation of C5 <sup>267</sup>. The functions of OmCI were initially investigated in its native non-glycosylated form, but also a hyper-glycosylated recombinant version was expressed, which demonstrated the same capacity to block the cleavage of C5a from C5 in the classical and alternative complement pathways. This inhibition could be competed with excess C5. But rather than acting on the C5 convertase, western blots, and gel filtration chromatography using labelled proteins demonstrated that this inhibition was mediated by direct binding of OmCI to C5, as subsequently confirmed by the crystal structure of the complex <sup>272</sup>. Further structural analysis of recombinant OmCI revealed that it additionally captured the inflammatory mediator leukotriene B4 (LTB4) in an internal binding pocket <sup>273,274</sup>. This bifunctionality, that is

inhibition of C5 and sequestering of LTB<sub>4</sub>, was investigated in a mouse model of immune complex-induced acute lung injury (IC-ALI). The treatment of IC-ALI mice with OmCI significantly attenuated the disease, suggesting a possibility for therapeutic application in humans suffering from immune complex-dependent diseases. Moreover, *in vivo* studies in a rat autoimmune myasthenia gravis model demonstrated efficacy of OmCI in the treatment of acute disease <sup>275,276</sup>. Further significance of OmCI for application in humans has been demonstrated by comparative *ex vivo* studies of human and pig blood, as well as pig *in vivo* studies, which suggested that OmCI could be successfully applied to humans <sup>277</sup>. Notably, it was shown in these experiments that OmCI inhibits all three complement activation pathways in *Escherichia coli*-induced sepsis. This kind of inflammation has been shown to depend on complement factors as well as the LPS-binding TLR4 co-receptor CD14 <sup>278</sup>. Therefore, more recent studies measured the decrease in inflammatory mediators, such as IL-1 $\beta$ , IL-8, and TNF- $\alpha$ , induced by a combination of OmCI and antibodies targeting CD14 <sup>279</sup>. This combination amplified the immune inhibitory effect of OmCI as compared to single administration. Under certain circumstances when only partial inhibition of the complement system is required, for example in the treatment of some forms of autoimmune diseases, transplant rejection, or cancer, the specific inhibition of the complement system in a terminal stage of the pathway, such as the cleavage of C5, may be of advantage since other functions of complement which mediate clearance of immune complexes and opsonisation, are not affected <sup>280–286</sup>. In the long run however, an imaginable disadvantage of OmCI administration might be that individuals could develop immunity against it. Hepburn and colleagues have addressed this possibility and injected OmCI daily into rats for seven days, but did not detect an immune response. This lends support to the assumption that salivary proteins from ticks are generally poorly

immunogenic in order to enable ticks to stay attached to their hosts for the completion of their feeding <sup>275</sup>.

#### *Lipocalins from the prostriate tick *Ixodes ricinus**

In prostriate hard ticks, 14 lipocalins from *Ixodes ricinus* (LIR) have been identified and characterised at once <sup>287</sup>. Their molecular weights vary from 21.8 kDa to 37.2 kDa and all appear to be glycosylated. Phylogenetic analysis of these LIR suggests however, that they belong to 6 distinct phylogenetic groups with potentially different functions. It was shown that LIR6 binds specifically to LTB<sub>4</sub>, an inflammatory mediator that is also bound by OmCI <sup>288</sup>. The overlap in function of two lipocalins from distinct tick families might suggest that LTB<sub>4</sub> is an important target for ticks in order to evade innate immune responses by the host. This would make sense since LTB<sub>4</sub> recruits and activates a number of immune cells such as neutrophils, eosinophils, mast cells, T cells, monocytes, and dendritic cells.

#### *Histamine-binding proteins from the metastriate tick *Rhipicephalus appendiculatus**

Histamine-binding lipocalins have been identified in SGE of the metastriate hard tick *Rhipicephalus appendiculatus*, the species in which also Japanin has been identified <sup>289</sup>. *R. appendiculatus*-derived histamine-binding proteins (Ra-HBPs) are proteins with a molecular mass of approximately 20 kDa that may form dimers when glycosylated. Histamine is mainly released by mast cells and basophils as an inflammatory mediator in response to tissue damage. Ticks cause tissue damage when they penetrate the skin during feeding. Hence, Ra-HBPs might represent a strategy to prevent histamine-mediated elimination and to feed successfully. The anti-inflammatory function of Ra-HBPs might be

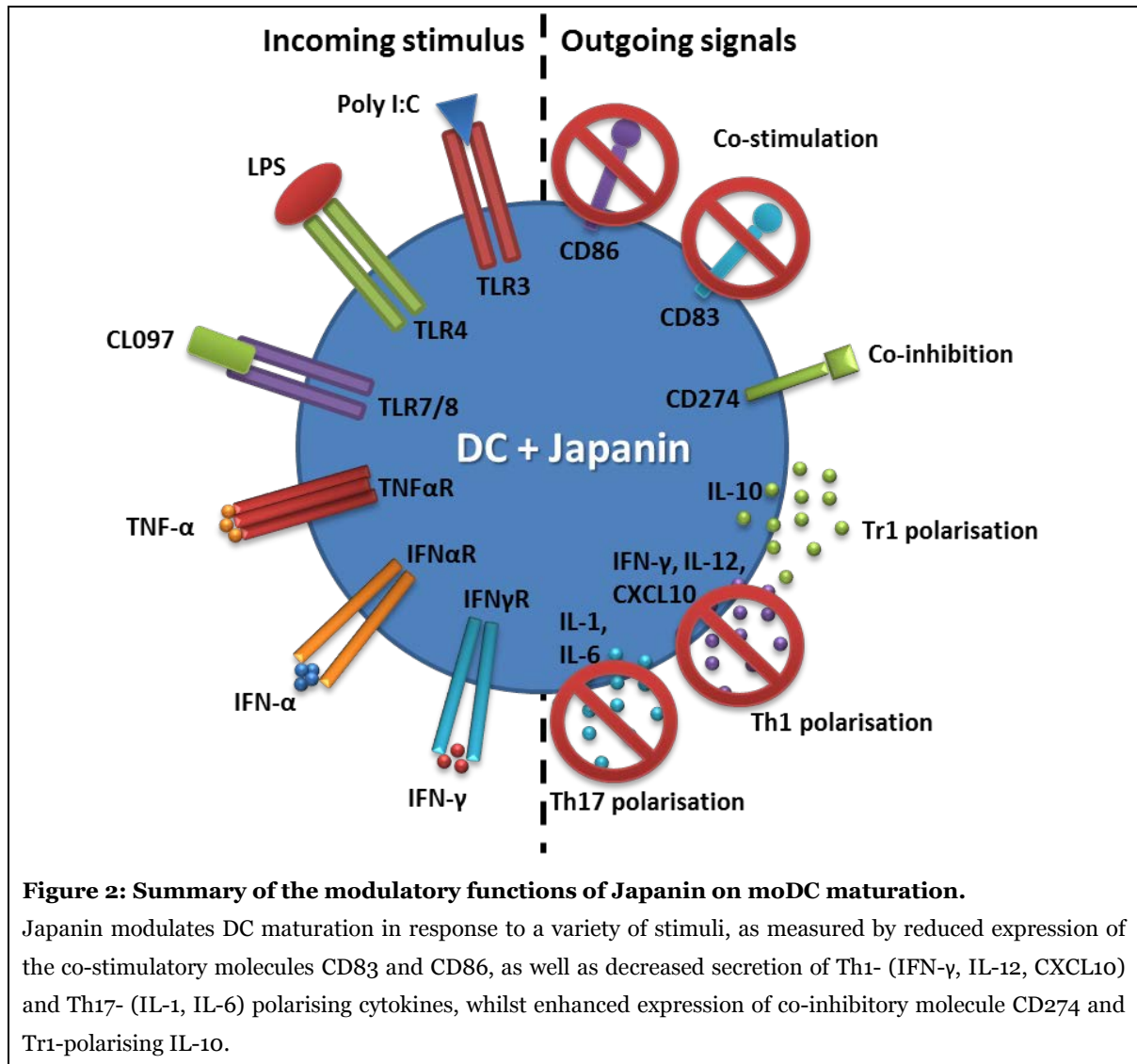
useful for therapeutic applications, for example in the treatment of allergies or histamine intolerance.

## 1.5 Japanin: a tick-derived DC-modulatory lipocalin

We have recently reported the identification of Japanin, a 17.7 kDa, N-glycosylated lipocalin with DC-modulatory functions, originally isolated from the brown ear tick *Rhipicephalus appendiculatus*<sup>290</sup>. *R. appendiculatus* belongs to the family of metastriate hard ticks and is a transmitter of pathogens, such as viruses or protozoa associated with severe disease in cattle, sheep and goat. The life cycle of *R. appendiculatus* involves three stages on three different hosts<sup>291</sup>. Larvae hatch from eggs and feed on one host, then drop to the ground and moult to nymphs. Nymphs may crawl onto plants, attach to another host and feed on it. Fed nymphs detach from their host, moult and develop into adult ticks. Adult ticks may climb plants in order to attach to another host and feed. Male ticks have several small blood meals before they mate and die. Female ticks on the other hand take one long feed on one host, lay a huge batch of eggs and then die<sup>292</sup>. The effects of Japanin were first observed in whole SGE of female *R. appendiculatus* ticks that had fed for 3 days on laboratory guinea pigs. For the production of SGE, salivary glands of the engorged ticks were dissected and disrupted. The crude extract was clarified from debris by centrifugation and then tested for activity on DC. *R. appendiculatus* SGE was found to impair maturation of DC in response to LPS, as measured by the expression of CD86. In order to identify the active component, SGE was separated by chromatography and screened for active fractions by the same measures. A protein was identified as the compound in SGE that impaired DC maturation. It was cloned and recombinantly expressed in a baculovirus system. 'Japanin' was termed after Jonathan M. Austyn and Patricia A. Nuttall as they discovered this lipocalin. Japanin has been shown to

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reprogramme immune responses of human moDC to a variety of stimuli, as measured by the expression of differentiation markers, co-stimulatory and co-inhibitory surface molecules, as well as the secretion of pro- and anti-inflammatory cytokines. The findings are summarised in Figure 2 and will be shown in more detail in the results chapters.



### 1.5.1 Identification of Japanin in SGE and cloning for recombinant expression

The initial screen for immunomodulatory activity in SGE from *R. appendiculatus* only examined the expression of CD86 by DC responses to LPS, since this TLR4 agonist represents the best investigated stimulus of DC. Immunogenic DC maturation is indicated

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within hours after stimulation with LPS by upregulation of the co-stimulatory surface molecule CD86. Incubation of DC with SGE prior to stimulation with LPS reduced the upregulation of CD86. This inhibitory effect was abrogated by the treatment of SGE with Proteinase K, showing that the active component was a protein. In order to purify this protein, SGE was firstly passed through an anion exchange chromatography column. Meanwhile the flow through was collected and subsequently separated by size-exclusion and thereafter by high performance liquid chromatography (HPLC). All fractions were screened for DC-modulatory activity with the assay described before. The most active fraction was sequenced by Edman degradation of N-terminal amino acids, allowing the generation of primers for RT-PCR cloning of a cDNA. The resulting 460 bp sequence was then used as a template for primers in a 5' RACE-PCR, eventually allowing cloning of the complete Japanin-encoding sequence (Genbank accession KC412662). This sequence consists of 531 bp, encoding a peptide of 152 amino acids plus a cleavable secretion signal of 24 residues. Based on the primary amino acid composition, the mature Japanin peptide has a predicted molecular weight of 17.7 kDa and conserved cysteine residues indicate that it is a member of the lipocalin family. Combined with a polyhistidine-tag for detection and purification purposes, the Japanin gene sequence was inserted into vectors of bacterial (pET52b), mammalian (pcDNA3.1), and arthropod (pBacPAK8) expression systems. Western blots probed with anti-polyhistidine antibodies showed that recombinant Japanin was produced by insect cells infected with Japanin-encoding baculovirus, as well as transfected mammalian cell lines, but not transformed bacteria. Given the origin of Japanin, an arthropod expression system seemed the most appropriate for subsequent experiments with the protein. Moreover, compared to expression in mammalian cells,

much higher yields could be obtained, which enabled the purification and concentration of recombinant Japanin stocks.

### 1.5.2 Japanin homologues

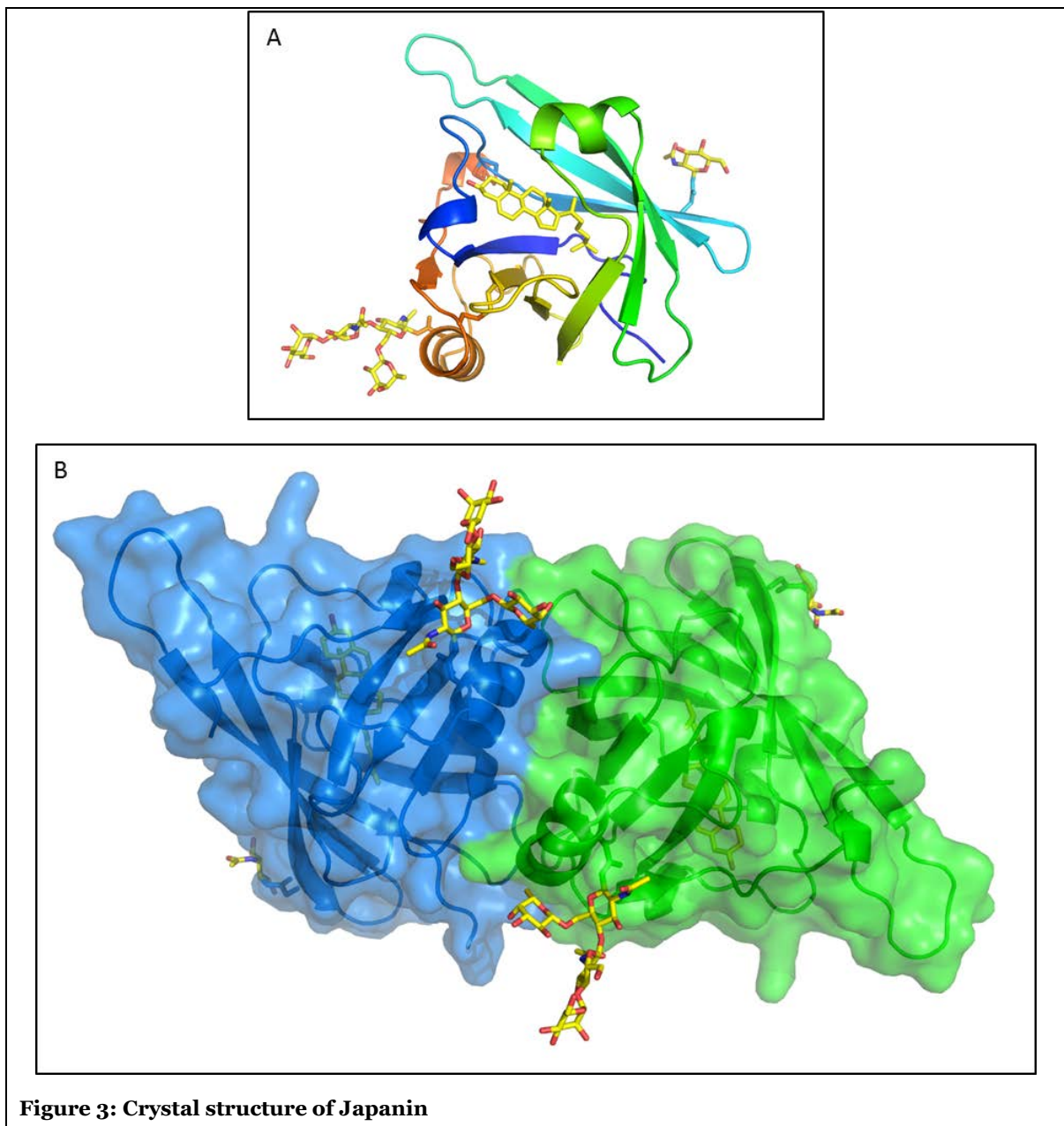
As a consequence of multiple gene duplication events during evolution, ticks often express different isoforms of a protein <sup>293</sup>. It was therefore investigated whether any Japanin-like proteins were encoded in the genomes of *R. appendiculatus* or the closely related species *R. sanguineus*. In order to identify putative homologues, degenerate primers were designed based on the sequence of Japanin and utilised in PCR with cDNA expression libraries of *R. appendiculatus* or *R. sanguineus*. Three proteins with significant similarity to Japanin resulted from this cloning approach: Two Japanin-like proteins derived from *R. appendiculatus*, JL-RA1 and JL-RA2 (Genbank accessions KC412664 and KC412665), and JL-RS from *R. sanguineus* (Genbank accession KC412663). All three homologues were found to have DC-modulatory activities. Notably, the efficacies correlated to the degree of similarity to Japanin, meaning the homologue protein with the most similar sequence showed the highest efficacy. Five other Japanin-related sequences were discovered in the genomes of metastriate ticks by search of public databases. Therefore we hypothesised that Japanin-like proteins might represent a distinct family of DC modulators that enable metastriate ticks to feed without provoking immune rejection mechanisms in their hosts.

### 1.5.3 The crystal structure of Japanin

Apart from immunobiological assays, purified recombinant Japanin has also been used for biochemical studies, leading to the recent resolution of the crystal structure of Japanin

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(manuscript in preparation; Pietro Roversi, Steven Johnson, Susan M. Lea). It shows that Japanin folds like a lipocalin, including an 8-stranded antiparallel  $\beta$ -barrel in the centre and an  $\alpha$ 2-helix between the last two strands (Figure 3 A). An additional  $\alpha$ 1-helix is located between strands  $\beta$ 5 and  $\beta$ 6. The crystal structure of Japanin was obtained in complex with cholesterol, inserted in the centre of the barrel and burying its aliphatic tail at the bottom of the pocket while binding to the central part with its ring regions. Moreover, the crystal structure of Japanin revealed that it forms homodimers (Figure 3 B).



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(A) Japanin in cartoon representation, coloured blue to red from N- to C-terminus. The disulphide bonds Cys28-Cys150 and Cys114-Cys138 are in stick representation and so are glycosylation sites Asn 35 and Asn131, the N-linked glycans on them, and the bound cholesterol molecule (C: yellow; N: blue; O: red). (B) Japanin dimer in surface representation with one monomer in green, one in cyan and the cartoon visible underneath. Images taken from Pietro Roversi, Steven Johnson, and Susan M. Lea; manuscript in preparation.

## 1.6 Aims and Objectives

### **I. Further investigation of the modulatory functions of Japanin**

- i. Production of purified and concentrated stocks of recombinant Japanin
- ii. Observation of the activity of Japanin in the presence of stimuli other than LPS
- iii. Examination of measures for the functions of Japanin other than CD86
- iv. Comparison of the effects of Japanin versus LPS on moDC differentiation
- v. Investigation of the role of Japanin in T cell activation in an MLR with DC

### **II. Identification of Japanin-binding cells and receptors**

- i. Determination of leucocyte populations that bind labelled Japanin
- ii. Isolation of Japanin-binding receptors by pull-down from DC
- iii. Confirmation of the binding by affinity assays with recombinant receptors
- iv. Verification of Japanin-binding receptors on DC by specific knock-down

### **III. Resolution of the mechanism of action of Japanin**

- i. Evaluation of the activity of Japanin on 'Japanin receptor'-negative cells
- ii. Optional investigation of additional modes of actions of Japanin

## 2 Materials and Methods

### 2.1 Mammalian cell culture

For maintenance, cell lines were incubated in 75 cm<sup>2</sup> (#4306141, Corning) or 175 cm<sup>2</sup> (#431080, Corning) tissue culture flasks at 37 °C and 5 % CO<sub>2</sub> in a humidified atmosphere, and sub-cultured once or twice a week (see Table 1) using aseptic techniques. Depending on the cell type, RPMI-1640 (#R0883, Sigma), high-glucose Dulbecco's Modified Eagle's Medium (DMEM, #E15-011, PAA), or Minimum Essential Medium Eagle Alpha Modification (αMEM, #M8042, Sigma) was used as a basal medium. All basal media were completed with foetal calf serum (FCS) (lot 0814, #A11-101, PAA; or lot 41Q7410K, #10270, Gibco), 2 mM L-glutamine (#M11-004, PAA), 100 IU/ml Penicillin and 100 µg/ml Streptomycin (#P11-010, PAA); additionally 50 µM 2-mercaptoethanol (2ME) (#M7522, Sigma) was added when required (Table 1). In order to detach adherent cells from the culture flasks for sub-cultivation, the supernatant was decanted with a pipette and the cell layer was washed with PBS/EDTA (#BE02-017F, Lonza) to remove any remaining medium. The adherent cell line 5637 was then incubated in PBS/EDTA plus trypsin (#L11-001, PAA) for about 5 min at 37 °C until cells detached. RAW264.7, DC2.4, R2, and PIEC-A8 were treated with PBS/EDTA only, because these cells were used to test the binding of Japanin and we wished to exclude potential damage to relevant surface molecules by trypsin. At least an equal amount of complete medium was added to neutralise EDTA and trypsin. In the case of DC 2.4, instead of fresh medium, the decanted supernatant was added back at this stage, because these cells only loosely attached to the flask and some came off already when decanting the first supernatant. Cells were then pelleted at 1200 rpm or 200-300 x g for 5 min and re-suspended in fresh medium.

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**Table 1: Cell lines**

Cell line	Origin	Medium	Split frequency	Density for seeding x 10 <sup>5</sup> /ml	Volume per 75 cm <sup>2</sup> in ml	Yield x 10 <sup>5</sup> /ml
<b>HL-60</b>	Human; acute promyelocytic leukaemia; promyeloblast	RPMI, 10 % FCS	Every 3 or 4 days	1	20	20
<b>U-937</b>	Human; histiocytic lymphoma	RPMI, 10 % FCS	Every 3 or 4 days	1	20	15
<b>THP-1</b>	Human; acute monocytic leukaemia; monocyte	RPMI, 10 % FCS, 2ME	Every 6-8 days	2	30	15
<b>Mutz-3</b>	Human; acute myelomonocytic leukaemia; monocytes	αMEM, 20 % FCS, 20 % 5637-conditioned medium (0.2 μm-filtered), 2ME	Every 4-6 days	2	20	15
<b>5637</b>	Human; renal cell carcinoma	RPMI, 10 % FCS, 2ME	Every 3 or 4 days for maintenance; Every 1 or 2 days for supernatant harvest	2 for maintenance 5 for supernatant harvest	13	20
<b>RAW 264.7</b>	Mouse, strain BALB/c; Abelson murine leukaemia virus-induced tumour; macrophage	DMEM, 10 % FCS	Every 3 or 4 days	0.5	10	25
<b>DC 2.4</b>	Mouse, strain C57Bl/6; dendritic cell	DMEM, 10 % FCS	Every 3 or 4 days	2	20	20
<b>WEHI-3</b>	Mouse, strain BALB/c; myelomonocytic leukaemia	RPMI, 10 % FCS	Every 3 or 4 days	1	10	10
<b>B95-8</b>	Marmoset; lymphocytes	RPMI, 10 % FCS	Every 3 or 4 days	1	10	10
<b>R2</b>	Rat; macrophages	RPMI, 10 % FCS	Every 3 or 4 days	1	10	10
<b>PIEC-A8</b>	Pig; iliac artery endothelial	RPMI, 10 % FCS	Every 3 or 4 days	1	10	10

### 2.1.1 Freezing and thawing of cell lines

For freezing, cells were centrifuged at 4 °C and aliquoted to  $1-5 \times 10^6$  cells per cryovial in 0.1-0.5 ml of their respective medium plus an equal volume of 20 % DMSO in FCS. Aliquots were cooled from +4 °C to -80 °C in a freezing container (Nalgene® Mr. Frosty, #C1562, Sigma) and then transferred into liquid nitrogen for long-term storage. For cell thawing, cryovials were incubated for 2-3 min in a waterbath at 37 °C and the suspension was then diluted in at least 10 ml of complete medium, to neutralize the toxicity of DMSO. Cells were then pelleted by centrifugation and re-suspended in fresh medium.

### 2.1.2 Mutz-3 differentiation into interstitial DC and Langerhans cells

For differentiation of interstitial DC, Mutz-3 were seeded at  $1 \times 10^5$ /ml in 5 ml per well of a 6-well tissue culture plate (#3516, Costar) and incubated for 7 days in complete  $\alpha$ MEM, containing 20 % FCS, plus recombinant human GM-CSF (#300-03, PeproTech) at 100 ng/ml (= 1000 IU/ml), TNF- $\alpha$  (#300-01A, PeproTech) at 2.5 ng/ml (= 120 IU/ml) and IL-4 (#200-04, PeproTech) at 20 ng/ml; cytokines were refreshed on day 3. For differentiation of Langerhans cells, Mutz-3 were cultured for 10 days in complete  $\alpha$ MEM, containing 20 % FCS, plus GM-CSF at 100 ng/ml (= 1000 IU/ml), TNF- $\alpha$  at 2.5 ng/ml (= 120 IU/ml) and TGF- $\beta$ 1 (#100-21, PeproTech) at 10 ng/ml; cytokines were refreshed on days 4 and 8.

## 2.2 Human PBMC isolation and moDC generation

For the isolation of peripheral blood mononuclear cells (PBMC) from blood of healthy human donors, leucocyte cones were obtained from the National Blood Service. Lymphoprep™ (#07811, Stemcell) was used as a density gradient medium in order to separate PBMC from granulocytes, erythrocytes, and platelets by centrifugation. Using

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aseptic techniques, ~7.5 ml fresh blood were diluted with Dulbecco's Phosphate Buffered Saline (PBS) (#D8537, Sigma) to a total volume of 35 ml and underlaid with 10 ml Lymphoprep in a 50 ml centrifuge tube. Centrifugation was performed at 800 x g for 30 min at room temperature, during which heavier erythrocytes and granulocytes sediment through the gradient medium to the bottom of the tube. Meanwhile, the lighter blood components remain above, and PBMC concentrate in an interface between and the gradient medium and the blood plasma at the top. The PBMC interface was harvested and washed twice in 50 ml PBS, containing 2 % FCS (lot 0814, #A11-101, PAA; or lot 41Q7410K, #1234567, Gibco) (PBS/FCS), by centrifugation at 400 x g and 300 x g, then washed once in 50 ml PBS/EDTA containing 2% FCS (PBS/EDTA/FCS) at 200 x g, to remove platelets and traces of plasma or lymphoprep. Eventually, the cells were resuspended in 5 ml PBS/EDTA/FCS.

For isolation of monocytes, the EasySep™ human monocyte enrichment kit (#19059, Stemcell) was used with a "The Big Easy" EasySep™ magnet (#18001, Stemcell) and 14 ml polystyrene tubes (#352057, DB Falcon) according to the instructions of the manufacturer. Monocytes were counted after incubation in red cell lysing buffer (#R7757, Sigma).

For generation of monocyte-derived dendritic cells (moDC), up to 60 ml of monocyte suspension were seeded at  $5 \times 10^5$ /ml into 75 cm<sup>2</sup> tissue culture flasks (#430641, Corning) and incubated for 6 days in complete RPMI-1640 (#R0883, Sigma) [i.e. supplemented with 10 % FCS (lot 0814, #A11-101, PAA; or lot 41Q7410K, #1234567, Gibco), 2 mM L-glutamine (#M11-004, PAA) and 100 IU/ml penicillin and 100 µg/ml streptomycin (#P11-010, PAA)], plus 100 IU/ml recombinant human GM-CSF (#300-03, PeproTech; or #04-rHuGM-CSF, Gentaur) and 500 IU/ml IL-4 (#200-04, PeproTech). Cytokines were

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refreshed on day 3 of culture by spinning down 1/3 of the volume at 1200 rpm or 200-300 x g for 5 min, and re-suspending the pelleted cells in complete RPMI plus a 3-fold concentration of GM-CSF and IL-4. MoDC were either used fresh for experiments, or frozen in Voluven® (6 % hydroxyethyl starch 130/0.4 in 0.9 % sodium chloride injection) plus 5 % DMSO and 7 % FCS and thawed (as described under 2.1.1) when needed.

## 2.3 Japanin production

### 2.3.1 Insect cell culture

Sf9 insect cell cultures were incubated at 28 °C in Sf-900™ III serum free medium (#12658-019, Gibco / Invitrogen) with 100 IU/ml Penicillin and 100 µg/ml Streptomycin (#P11-010, PAA), shaking at 150 rpm in Erlenmeyer flasks with cellulose stoppers. Cells were sub-cultivated twice a week by seeding  $2 \times 10^5$ /ml in a fresh flask, which was about a 1/10 spilt. Erlenmeyer flasks were filled to only 1/5 – 1/4 of the total volume capacity to ensure optimal cell and oxygen distribution.

### 2.3.2 Baculovirus transfection and amplification

For transfection with baculovirus, Sf9 were seeded in a 6-well plate at  $0.8-1 \times 10^6$  in ~2 ml per well and allowed to attach for 1 h. The medium was removed and 2.5 ml Sf-900™ III serum free medium without antibiotics was added. DNA-complexes were prepared for each transfection sample in antibiotic-free medium in a well of a 96-well plate by pipetting 5 µl Cellfectin II reagent (# 10362-100, Invitrogen) in 100 µl Sf9 medium and adding 40-100 ng flashBAC GOLD baculovirus expression vector (#100201, Oxford Expression Technologies) in 2-5 µl and 500 ng Japanin cDNA. This mixture was firstly incubated for 15-30 min at room temperature, then pipetted drop wise to the seeded cells and incubated

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at 28 °C for 5 days in a humidified atmosphere. Cell supernatants were harvested as recombinant baculovirus stock suspension by centrifuging for 10 min at 2000 rpm at 4 °C. Stocks were frozen for storage at -80 °C. For virus amplification, Sf9 cells were seeded in log phase at  $1 \times 10^6$ /ml, inoculated with virus stock at a 1:1000 ratio or at an moi = 0.1, if virus titre was known. Virus-inoculated cells were incubated for 3-4 days shaking at 150 rpm in Erlenmeyer flasks with cellulose stoppers filled to 1/5 – 1/4 of the total capacity. When cells appeared more granular and enlarged compared to non-inoculated cultures, supernatants were collected after centrifugation of the suspension for 10 min at 2000 rpm at 4 °C and the virus harvest was frozen for storage at -80 °C.

Virus titres were determined by plaque assay. 2 x Grace's Medium (#11667-037, Gibco) was supplemented with 20 % FCS (lot 41Q7410K, #10270, Gibco) and warmed to at least room temperature or ideally placed in a waterbath at 37 °C. Sf9 cells were used in log phase, i.e.  $<3 \times 10^6$ /ml, and diluted to  $5 \times 10^5$ /ml in Sf900III medium before seeding  $10^6$  cells in 2 ml per well of a 6-well plate. Cells were allowed to settle and adhere for about 30 minutes. Virus was diluted in subsequent 10-fold dilutions, up to a final dilution of  $10^{-6}$ , or  $10^{-7}$ , depending on the expected virus titer. The cell culture medium was removed from the cells in the plate and 1 ml diluted virus was added per well for every dilution to be tested. The assay was incubated on a slowly rotating platform for 2-4 hours. After this incubation the supernatant was removed and replaced with 3 ml per well of a mix of 1 part 2 x Grace's medium with 20 % FCS and 1 part sterile 3 % low-melting agarose (#50101, Lonza) in H<sub>2</sub>O, warmed to 37 °C. Plates were left to set for about 30 minutes, then transferred into a moist incubator at 28 °C. After 4-6 days the assays were stained by adding 1 ml of 0.01 % neutral red in Grace's medium-FCS-agarose mix per well and plaques were counted after another 4-24 hours incubation at 28 °C. Since 1 ml virus stock had been added per test

well, the virus titre in pfu/ml could be calculated for each dilution with countable plaques from the plaques per well; e.g. 56 plaques in a  $10^{-6}$  dilution resulted in a virus titer of  $5,6 \times 10^7$  pfu/ml.

### 2.3.3 Protein expression and purification

#### *Inoculation of Sf9 with baculovirus*

Sf9 cells were seeded at  $1 \times 10^6$ /ml and cultured overnight shaking at 28 °C in Erlenmeyer flasks, filled to no more than 1/5 – 1/4 of the total volume capacity. Cells were inoculated with Japanin-encoding baculovirus the next day, when they reached  $2 (\pm 0.5) \times 10^6$ /ml, at an moi of 1.5-2 pfu/cell and incubated for another 4 days.

#### *Japanin harvest*

Inoculated Sf9 cultures were centrifuged for 10 min at 2000 rpm (ca. 800 x g) to remove cells and debris. The Japanin-containing supernatant was collected and slowly mixed on ice with PEG 6000 (#81260, Sigma Aldrich) to a final concentration of 15 %, by adding 18 % of the supernatant weight, and incubated at 2-8 °C overnight. The mixture was then centrifuged at 4 °C for 30 min at ca. 10000 x g (10000 rpm Beckman centrifuge model J2-21, rotor JA-10). The PEG supernatant was discarded and the pellet was thoroughly resuspended in 10 ml HBSS with  $\text{Ca}^{2+}$  (#H15-008, PAA) and incubated on a rotating platform or roller at room temperature for 2-4 hours or at 2-8 °C overnight. The protein suspension was then centrifuged again at 4 °C for 30 min at ca. 10000 x g (10000 rpm J2-21, rotor JA-10) and the Japanin-containing supernatant was decanted and 0.22  $\mu\text{m}$ -filtered into a sterile vessel.

### *Automated TALON purification*

HisTALON™ Superflow Cartridges (#635650, Clontech) were connected to a BioLogic DuoFlow chromatography pump (Bio-Rad). The maximal pressure was set to 72 psi and the flow rate to 1 ml/min. Firstly, the TALON™ column was washed with 5 ml distilled water, to remove the storage buffer, then equilibrated with 10 ml HBSS. Next the Japanin harvest was loaded. Afterwards the column was washed with 15 ml TALON™ wash buffer (40 mM sodium phosphate, 300 mM sodium chloride, 5 mM imidazole, 10 % glycerol, pH 7.4). His-tagged proteins were eluted with 8 ml TALON™ elution buffer (40 mM sodium phosphate, 300 mM sodium chloride, 150 mM imidazole, pH 7.4) and collected in 1 ml fractions in Eppendorf® Protein LoBind microcentrifuge tubes (#Z666505, Sigma Aldrich). Since the column was reusable, it was afterwards washed again with TALON™ wash buffer and distilled water and stored in 20 % ethanol until the next use.

### *Size Exclusion Chromatography (SEC)*

The TALON™ eluate was concentrated to 100-200 µl using spin filters with a molecular weight cut-off of 9000 (#87748, Thermo Scientific). For separation of Japanin from any other proteins that might have bound to the TALON™ column, a superdex® size exclusion column (GE Healthcare Life Sciences) was attached to the chromatography pump. The maximal pressure was set to 261 psi and the flow rate to 0.5 ml/min. Firstly, the injection port and loading loop of the chromatography pump were briefly flushed with distilled water and the column was washed with 30 ml distilled water. Thereafter the system was equilibrated in the same way with PBS, until the increase in conductivity reached a plateau. Then the concentrated TALON™ eluate was injected into the 500 µl loading loop and pumped over the superdex® column with PBS at 0.5 ml/min for at least 30 min. UV

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peaks were collected as fractions of 0.5 ml in Eppendorf® protein LoBind microcentrifuge tubes. When the elution was complete, the system was washed with distilled water until the conductivity decreased to zero, and then stored in 20 % ethanol.

The Japanin protein concentration of size exclusion chromatography (SEC) fractions was determined by nanodrop analysis, measuring the absorption at 280 nm with an extinction coefficient of 20 %. The purity of Japanin in the fractions was then analysed by silver staining of an SDS-PAGE (see 2.4) with 50 ng of each per lane. Pure Japanin fractions were pooled and sterile-filtered using 0.2 µm 1.5 ml Corning® Costar® Spin-X® centrifuge tube filters (#CLS8160, Sigma Aldrich) and stored at -20 °C or -80 °C for long term storage, or in the fridge for short term storage.

#### *Fluorescent protein labelling for FACS*

DyLight 649 NHS-Ester (#46416, Thermo Scientific) was used for fluorescent labelling of Japanin and the control protein OmCI as recommended by the manufacturer. Purification columns were made of Biogel P-6 fine resin in spin filters from another microscale protein labelling kit (component E+D of #A30006, Invitrogen). Protein recovery was determined by nanodrop analysis as before.

#### *Protein labelling with biotin*

For labelling of Japanin or OmCI with biotin, EZ-Link Sulfo-NHS-SS-Biotin (#21328 or #21326, Thermo Scientific) was applied at a 30-fold excess, according to the instructions of the manufacturer.

## 2.4 SDS-PAGE

Protein samples for SDS-PAGE were mixed with 4 x LDS loading buffer (#NP0007, Invitrogen; or #84788, Thermo Scientific) and 50  $\mu$ M DTT (#43816, Sigma), and heated to 70 °C for 10 min in a DNA Engine® Thermal Cycler (#PTC-200, Bio-Rad) before loading onto a protein gel with a 4-20 % polyacrylamide gradient (#25224, Thermo Scientific). Electrophoresis was conducted in an XCell SureLock™ Mini-Cell (Invitrogen) at 100-120 V for 45-60 min at room temperature or 2-8 °C. Running buffer was made up from a 10 x HEPES/Tris/SDS stock, containing 119 g HEPES (#H3375, Sigma), 60.5 g Trizma Base (#T6066, Sigma), and 5 g SDS (#L4509, Sigma) in 500 ml.

### 2.4.1 Western Blotting

SDS gels were blotted on PVDF (Immobilon-P<sup>®</sup>, #ISEQ00010, Millipore) or nitrocellulose (#RPN203D, GE Healthcare) membranes between gel blot papers (#10427813, Whatman) and sponges in a 1 l or 2.5 l tank (Bio-Rad) with transfer buffer (3.03 g/l Trizma Base, 14.46 g/l Glycine, 20 % methanol) at 2-8 °C at 400 mA for 1 hour or 80 mA overnight. Membranes were blocked for at least 30 min in StartingBlock™ T20 (#37539, Thermo Scientific) or Carbo-Free™ blocking solution (#SP-5040, Vector Laboratories) before incubation with antibodies or peroxidase-conjugated streptavidin. For staining of specific protein bands, membranes were incubated in primary antibodies for 1 hour at room temperature or overnight at 2-8 °C, in secondary antibodies for 30-60 min at room temperature, and in streptavidin for 30 min at room temperature. Primary antibodies were made up in blocking solution and reused, secondary antibodies and streptavidin were diluted in washing buffer and discarded after one use. Membranes were washed after each staining in PBS containing 0.05 % Tween<sup>®</sup>20 (#P5927, Sigma)

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3 times for 5 min and once for 15 min at room temperature. Pierce® enhanced chemiluminescence (ECL) western blotting substrate (#32106 Thermo Scientific) was used to visualise targeted protein bands on X-ray film (#PN34090, Thermo Scientific).

Japanin was detected by biotin tag by peroxidase-conjugated streptavidin (#016-030-084, Jackson ImmunoResearch Laboratories) diluted 1:20000 in PBST from 1 mg/ml stock, or by his tag by biotin-conjugated mouse anti-6 x his antibodies (#ab106261, abcam) diluted 1:10000 in StartingBlock™ T20 plus goat anti-mouse Ig antibodies conjugated to horseradish peroxidase (HRP) (#P0447, Dako) diluted 1:1000 in PBST. CD206 was detected by goat anti-human MMR/CD206 (#AF2534, R&D systems) diluted 1:5000 in blocking solution, plus donkey anti-goat IgG-HRP (#sc-2020, Santa Cruz Biotechnology) diluted 1:5000 in washing buffer. Carbo-Free™ blocking solution (#SP-5040, Vector Laboratories) and a PBST washing buffer containing 0.5 M NaCl were used in the detection of CD206 in order to reduce background binding of the antibodies. These reagents were also used for the detection of stabilin-1 by sheep anti-human stabilin-1 (#AF3825, R&D systems) 1:500 plus donkey anti-sheep IgG-HRP 1:10000 (#A 3415, Sigma). Phosphorylated STAT3 was detected by rabbit anti-phospho-Stat3 (Tyr705) (D3A7) (#9145, Cell Signaling) plus goat anti-rabbit IgG-HRP (#7074, Cell Signaling).

#### 2.4.2 Silver Staining

Protein bands in SDS gels were visualised using a Pierce® silver stain kit (#24612, Thermo Scientific) according to the instructions of the manufacturer.

## 2.5 Deglycosylation of Japanin

### 2.5.1 Restriction digest and ligation of vectors for double-glyco mutants

Mutated versions of Japanin in which the glycosylation motifs were destroyed had already been cloned in our lab for expression in mammalian cells. For transfection of insect cells in the study at hand, these altered Japanin sequences were cut out of the mammalian vectors and inserted into baculovirus vectors. Therefore the following “pcDNA 3.1 jap his” constructs were used:

- N35D-N131D
- N35D-T133A
- T37A-N131D
- T37A-T133A

A master mix for the restriction digest was made up of 15 µl Buffer 4 (10x, #B7004S, New England Biolabs (NEB)), 1.5 µl purified bovine serum albumin (BSA) (10 mg/ml = 100x, #B9001S, NEB), 2.5 µl BamHI (20000 U/ml, #R3136S, NEB), 2.5 µl NotI (20000 U/ml, #R3189S, NEB), and 113.5 µl distilled water; 27 µl master mix were added to 1 µg DNA. Samples were incubated in a DNA Engine® Thermal Cycler (#PTC-200, Bio-Rad) at 37 °C for 1 hour. Samples were mixed with 6 µl blue gel loading dye (6x, #370215, NEB) and loaded onto a 1.4 % agarose gel (molecular grade, #BIO-41025, Bionline) made up in running buffer, containing GelRed™ stain (41003, Biotium) 1:20000. 5 µl Hyper Ladder IV (lot H4-111B, # BIO-33056, Bionline) was used as a reference for DNA size. The electrophoresis ran at 100 V for 30 min. DNA bands were visualised with an UV lamp and those matching the insert size (0.6 kb) were cut out of the gel and weighed for the following extraction procedure. DNA was purified from the agarose gel using an illustra™

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GFX™ PCR DNA and gel band purification kit (#28-9034-70, GE Healthcare). Insert DNA was eluted in 30 µl. For DNA ligation, 50 ng pBacPAK8 vector (5.5 kb, GenBank accession #U02446) were used and inserts added in a 2:1 ratio in 10 µl reactions: 1 µl 10 x buffer for T4 DNA ligase (#B0202S, NEB), 0.5 µl T4 DNA ligase (#EL0011, Fermentas), 1 µl pBAC PAK8 BamHI-NotI at 55 ng/ml, 6.5 µl purified insert DNA, 1 µl distilled water; a reaction without insert DNA was set up as a negative control. The ligation was incubated at room temperature for ~30 min.

For amplification of the new construct, 5 µl ligation were added to 50 µl competent *E. coli* (#C3019, NEB) and the transformation was incubated for 5-10 min on ice, then for 30 s in a waterbath at 42 °C. After the heat shock, 200 µl super optimal broth with catabolite repression (SOC) outgrowth medium (supplied with the bacteria) were added and incubation continued at 37 °C on a shaker for 45-60 min. Transformed bacteria were plated on 1.5 % agar (#A5054, Sigma), containing 2.5 % Miller's Luria Broth (LB) (#L3522, Sigma) and ampicillin as a selection antibiotic. Plates were incubated at 37 °C overnight. Two colonies of each mutant were picked and incubated in liquid LB medium with ampicillin shaking at 37 °C overnight. Plasmids were extracted from the cultures using a miniprep kit (#D4019, Zymo Research) and elution buffer without EDTA (#400721-19, Stratagene). DNA concentrations were determined by nanodrop and samples were sent for sequencing analysis to confirm the cloning success before using the plasmid vector containing mutated Japanin for transfecting Sf9 with the baculovirus.

### 2.5.2 PNGase F treatment

1 µg Japanin was incubated in 10 µl denaturing buffer at 100 °C for 10 min, then mixed with 2 µl G7 reaction buffer, 2 µl 10 % NP-40 and 1 µl PNGase F (#P07045 or P7055, NEB)

in a total reaction volume of 20 µl (adjusted with distilled water) and incubated in a DNA Engine® Thermal Cycler (#PTC-200, Bio-Rad) at 37 °C for 1 hour.

### 2.5.3 Con A purification

We attempted to purify deglycosylated Japanin after the treatment with PNGase F by binding cleaved glycans and potentially remaining glycosylated proteins to a concanavalin A (Con A) chromatography column. Deglycosylated Japanin was expected in the flow-through, while free glycans and glycosylated protein would bind to the column until they were released by an elution buffer high in mannose. For ideal binding conditions to the Con A column, a binding buffer was made up of 20 mM Tris, 0.5 M NaCl, 1 mM MnCl<sub>2</sub>, and 1 mM CaCl<sub>2</sub> and the pH was adjusted to 7.4 with HCl. Con A elution buffer contained 0.5 M methyl- $\alpha$ -D mannopyranoside, 20 mM Tris, and 0.5 M NaCl, and the pH was adjusted to 7.1 with HCl. Buffers were degased by stirring for 1 hour and 0.2 µm-filtered before use. 9 µg Japanin were treated with PNGase F as described above (2.5.2). A HiTrap Con A 4B column (#28-9520-85, GE Healthcare) was connected to the BioLogic DuoFlow chromatography pump (Bio-Rad) and washed with 30 ml distilled water. The column was equilibrated with 10 ml binding buffer, running at 1 ml/min. 10 µl PNGase F-treated Japanin were mixed with 490 µl binding buffer and injected into the chromatography loading loop. The sample was applied to the column at a flow rate of 0.1 ml/min over 50 min. During the entire run, fractions of 0.5 ml were collected. The UV detector showed a peak over fractions 2-5. Afterwards 5 ml elution buffer were pumped over the column at the same flow rate and fraction collection settings. No distinct peaks appeared, but the optical density increased conversely to the conductivity from fraction 4, showing when the elution buffer entered the system. The peak flow-through fractions and

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elution fractions 4-10 were concentrated in a spin filter with a molecular weight cut-off of 9000 (#87748, Thermo Scientific) to about 100 µl for subsequent analysis by SDS-PAGE and western blot or silver staining (see 2.4). Opposed to the eluate, the flow-through concentrate showed precipitate after the concentration. Traces of glycosylated Japanin could be detected in the eluate by anti-his tag antibodies on a western blot. PNGase F was apparently present in the flow-through, as shown by silver staining. However, there was no sign of deglycosylated Japanin, hence we concluded that it became insoluble by the treatment and formed the precipitate that had become visible in the flow-through after concentration. Unfortunately, it was impossible to detect any Japanin in non-concentrated flow-through, because the dilution factor was too high.

## 2.6 Japanin activity and activity inhibition assays

### 2.6.1 Surface molecule expression assay

Monocyte-derived DC or cell lines were concentrated to  $0.5-1 \times 10^6$ /ml and 100 µl cell suspension were seeded per well of a flat-bottom 96-well tissue culture plate. Japanin was added to 500 ng/ml in a total culture volume of 200 µl per well. Cells were either stimulated at the same time or after 18-24 hours incubation at 37 °C and 5 % CO<sub>2</sub> in a moist environment. Either 100 ng/ml LPS from *Escherichia coli* O55:B5 (#L4005, Sigma), 25 µg/ml poly I:C (LMW, #tlrl-picw, InvivoGen), or 4 µg/ml CLO97 (#tlrl-c97, InvivoGen) were used as stimulants. Cells were stained for flow cytometry 16-24 hours (typically 18 hours) after stimulation. The staining was conducted as described under 2.7.1. Differentiation and maturation markers were stained by the following fluorescently labelled antibodies against human antigens: CD1a FITC (#11-0019, eBioscience), CD14 PE (#12-0149, eBioscience), CD86 V450 (#560359, BD Horizon™), CD86 (B7-2) Alexa

Fluor® 488 (#53-0869, eBioscience), CD86 (B7-2) PE (#12-0869, eBioscience), CD274 (B7-H1) PE-Cy7 (#25-5983, eBioscience).

For activity inhibition studies, antibodies against human CD18 (#302111, BioLegend), IL-10 (#MAB2171, R&D Systems) or the IL-10R (#MAB274, R&D Systems) were added at the same time as Japanin; as were the chemical endocytosis inhibitors chloroquine, filipin III, latrunculin A, and chlorpromazine.

### 2.6.2 TNF- $\alpha$ ELISA

Culture supernatants from 2.6.1 were screened for TNF- $\alpha$  using murine or human TNF- $\alpha$  ELISA development kits (#900-K54, or #900-K25, PeproTech) according to the instructions of the manufacturer. Materials not included in the kit were utilised as described under 2.7.2.

### 2.6.3 Signalling assay

Monocytes or moDC were isolated and cultured as described under 2.2. Cells were seeded at  $10^6/100 \mu\text{l}$  per well of a 96-well tissue culture plate. Japanin was added to  $5 \mu\text{g/ml}$  in a final volume of  $200 \mu\text{l}$  per well. As negative controls, PBS and  $100 \text{ nM}$  of the WKYMVm (Trp-Lys-Tyr-Met-Val-(D-Met)-NH<sub>2</sub>) –peptide were added. WKYMVm has been shown to induce the phosphorylation of ERK, but not STAT3. After a 7 minute incubation time at  $37^\circ\text{C}$ , the cultures were placed on ice, the cell suspension immediately transferred into  $1.5 \text{ ml}$  Eppendorf microfuge tubes with chilled PBS and centrifuged for 5 min at  $300 \times g$  at  $4^\circ\text{C}$ . The supernatant was discarded and the cell pellet was lysed by resuspension in  $90 \mu\text{l}$  lysis buffer, consisting of  $25 \text{ mM}$  Tris-HCl at pH 7.6,  $150 \text{ mM}$  NaCl,  $1\%$  NP-40,  $1\%$  sodium deoxycholate, and  $0.1\%$  SDS, supplemented with the phosphatase inhibitors

sodium orthovanadate (1 mM) and Halt™ phosphatase inhibitor cocktail, and the protease inhibitors PMSF (1 mM) and Halt™ protease inhibitor cocktail. The lysate was immediately snap-frozen on dry ice and stored at -20 °C. For analysis, samples were thawed on ice, centrifuged in a microfuge at 13500 rpm for 10 min at 4 °C and the supernatant was prepared for SDS PAGE as described under 2.4. Western blots were probed with rabbit antibodies against phospho-Stat3 (Tyr705) (D3A7) (#9145, Cell Signaling) plus HRP-linked goat anti-rabbit IgG antibodies (#7074, Cell Signaling).

#### 2.6.4 T cell isolation and MLR

PBMC were isolated as described under 2.2 and specific T cell subsets were negatively selected as recommended in the instructions of immunomagnetic EasySep™ enrichment kits from Stemcell, specific for human naïve CD4<sup>+</sup> (#19155) and CD8<sup>+</sup> (#19158), or memory CD4<sup>+</sup> (#19157) and CD8<sup>+</sup> (#19159) T cells. These kits distinguish naïve and memory T cells by differential expression of CD45 isoforms: Naïve and effector T cells express CD45RA, memory T cells express CD45RO. Directly after isolation, T cells were stained with carboxyfluorescein succinimidyl ester (CFSE). Therefore, cells were firstly washed with HBSS and then resuspended in 2 ml RPMI without supplements but 10 µM CFSE (#65-0850-85, eBioscience). Cells were incubated with stain in a waterbath at 37 °C for 10 min. Afterwards cells were washed with complete culture medium and recounted before seeding. CD4<sup>+</sup> and CD8<sup>+</sup> T cells were mixed in a 1:1 ratio and 100 µl suspension were seeded at 5 x 10<sup>5</sup>/ml per well in a 96-well tissue culture plate. The final culture volume was made up to 200 µl with a suspension of moDC (isolated and cultured for 6 days as described under 2.2) at 5 x 10<sup>4</sup>/ml or culture medium alone, depending on the condition to be tested. LPS from Escherichia coli 055:B5 (#L4005, Sigma) and/or Japanin

were added at the same time at a concentration of 100 ng/ml or 500 ng/ml respectively. The cultures were incubated at 37 °C and 5 % CO<sub>2</sub> for 2, 5, 7, or 10 days. During the last 5 hours, cells were stimulated with 10 µg/ml brefeldin A (BFA) (#B5936, Sigma), 1 µM ionomycin calcium salt from *Streptomyces conglobatus* (#I0634, Sigma), and 20 ng/ml phorbol 12-myristate 13-acetate (PMA), in order to block protein transports and enhance intracellular cytokine accumulation. Intact cells were then stained with a mix of fluorescently labelled antibodies against surface molecules, followed by staining with fixable viability dye eFluor® 660 (#65-0864-14, eBioscience), as described under 2.7.1. All antibodies against surface molecules and corresponding isotypes were from eBioscience: anti-CD4 APC-eFluor® 780 (#47-0048), anti-CD8 PE Cy7 (#25-0088), anti-CD209 APC (#17-2099), mouse IgG1-κ APC-eFluor® 780 (#47-4714), mouse IgG1-κ PE Cy7 (#25-4714), mouse IgG1-κ APC (#17-4714). After the surface staining, cells were incubated for 30 min in 100 µl fixation/permeabilisation buffer (#00-5123-43 and #00-5223-56, eBioscience) and then washed twice with permeabilisation buffer (#00-8333-56, eBioscience) before incubation with antibodies specific for intracellular cytokines. Except for the Brilliant Violet 421™ anti-human IL-4 antibody (#500826, BioLegend) and corresponding rat IgG1-κ isotype control (#400429, BioLegend), all antibodies were purchased from eBioscience: anti-INF-γ PerCP-Cyanine 5.5 (Cy5.5) (#45-7319), anti-IL-17A PE (#12-7178), mouse IgG1-κ PerCP-Cy5.5 (#45-4714), mouse IgG1-κ PE (#12-4714).

### 2.6.5 Japanin-conditioned media studies

Japanin was added to 1 µg/ml in 1 ml of the following conditions in an untreated 24-well plate (#3738, Costar):

1. Confluent THP-1 suspension

2. 0.45 µm-filtered supernatant of a confluent THP-1 suspension
3. Complete RPMI, containing 10 % FCS, L-Gln, and Pen/Strep
4. HBSS containing 2 % FCS
5. PBS (negative control)
6. PBS (positive control)

Japanin was incubated in each condition for 24 hours at 37 °C. Condition 1, Japanin in cell suspension, was 0.45 µm-filtered after the incubation. 250 µl magnetic TALON™ beads (#635636, Clontech) were washed 4 times with 1 ml sterile PBS, then re-suspended in 250 µl PBS. 50 µl bead suspension were added to each condition except the PBS positive control and incubated for 5 hours on a rotator at 2-8 °C. The beads were pulled-out of the conditions using a magnet and all samples were sterilised with a 0.2 µm spin-filter (#CLS8160, Sigma Aldrich). 100 µl of each condition was mixed with 100 µl of an moDC suspension at 10<sup>6</sup>/ml in a 96-well tissue culture plate in the presence or absence of LPS. The activity of Japanin was assessed next to freshly diluted Japanin by measuring the expression of CD86 after an 18 hour incubation, following the procedure described under 2.6.1.

## **2.7 Japanin binding and binding inhibition assays**

### **2.7.1 Flow cytometry staining**

In order to visualize the binding of Japanin to cells via flow cytometry, fluorescently labelled Japanin or biotinylated Japanin (see 2.3.3) plus a Streptavidin-PE-Cyanine 7 (Cy7) conjugate (#25-4317, eBioscience) were used. Cells were usually concentrated to 0.5-1 x 10<sup>6</sup>/ml and 100 µl suspension was seeded into a well of a 96-well plate with round bottoms (Costar) on ice. The plates were centrifuged at 4 °C for 2 min at 1500 rpm. The

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supernatant was discarded and cells were resuspended in 200 µl HBSS with Ca<sup>2+</sup> (#H15-008, PAA) supplemented with 2 % FCS. Cells were then spun down again as before. After this wash step, cells were resuspended in 50 µl labelled Japanin or OmCI typically diluted in buffer to 1 µg/ml, and incubated for 45-60 min on ice. Before analysing the binding of Japanin on a BD FACS Canto II flow cytometer, cells were washed twice as before. Samples were either analysed directly after the staining was complete, or fixed in 4 % formaldehyde and analysed the next day. Flow cytometry data were evaluated with an up-to-date version of the analytical software FlowJo.

For binding competition or inhibition studies, graded doses of mannan from *Saccharomyces cerevisiae* (#M7504, Sigma) or anti-human CD18 antibodies (#302111, BioLegend) were added to the labelled Japanin suspension. The binding of CD18 antibodies was confirmed by staining with secondary goat anti-mouse IgG antibodies (#35508, Thermo Scientific).

For phenotyping of PBMC, in addition to Japanin or OmCI, cells were stained in the presence of an Fc receptor blocking reagent (#120-000-442, Miltenyi Biotec) with a mix of the following antibodies against human antigens: CD45-eFluor® 605 VC (#93-0459, eBioscience); biotinylated lineage markers: CD3 (#317320, BioLegend), CD7 (#13-0079, eBioscience), CD19 (#13-0199, eBioscience), CD20 (#13-0209, eBioscience), CD56 (#318320, BioLegend), plus Streptavidin-Alexa-Fluor 700 (#521383, Invitrogen); HLA-DR-V500 (#561224, BD Bioscience); CD14-Brilliant Violet 650™ (#301835, BioLegend); CD16-Brilliant Violet 711™ (#302043, BioLegend); CD1c-Brilliant violet 421 (#331526, BioLegend); CD123-PerCP-Cy5.5 (#306016, BioLegend); CD11b-PE-Cy7 (#301322, BioLegend); CD141-PE (#130-090-514, Miltenyi Biotec); CD11c-PE-Texas Red (#562392,

BD Bioscience). Dead cells were stained by fixable viability dye eFluor® 780 (#65-0865, eBioscience). This 12-colour staining was analysed on a BD™ LSR II flow cytometer.

### 2.7.2 Plate binding assay with recombinant CD206

Recombinant human CD206 (#2534-MR/CF, R&D systems) was immobilised on MaxiSorp® flat-bottom 96 well plates (#442404, Nunc) by incubating 100 µl per well of receptor suspension at 1 µg/ml in PBS (#D8537, Sigma) at 2-8 °C overnight. Plates were washed 4 times with 300 µl per well of PBS plus 0.05 % Tween 20, using a Skan Washer 400 (#12019, Skatron Instruments). For blocking of unspecific binding to potentially uncoated areas, 300 µl of a 3 % bovine serum albumin (BSA) (#A7906, Sigma) solution in HBSS (#H15-008, PAA) were added per well and incubated for 1 hour at room temperature. Plates were washed again, then incubated for 1 hour in successive dilutions of biotinylated Japanin (see 2.3.3) in blocking reagent. After another washing step, 100 µl per well of peroxidase-conjugated streptavidin (#016-030-084, Jackson ImmunoResearch Laboratories) diluted 1:5000 in blocking solution were added and incubated for 1 hour at room temperature. Plates were washed again before 100 µl 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (#A3219, Sigma) were added per well to visualise peroxidase activity. The absorption was measured at 405 nm in 5 min intervals for 25 min in a microplate luminometer (Lucy 1, Anthos Labtec Instruments).

For competition studies, the Japanin incubation occurred in the presence of graded doses of mannan from *Saccharomyces cerevisiae* (#M7504, Sigma) or D-mannose (#M6020, Sigma Aldrich).

### 2.7.3 Biacore analysis with recombinant CD206

The Biacore system (Biacore 3000, GE Healthcare) was operated with help from Marcus Bridge at the Sir William Dunn School of Pathology, University of Oxford. Firstly, optimal pH conditions for binding of recombinant CD206 to a CM5 sensor chip (BR-1003-99, GE Healthcare) were tested with soluble murine full length CD206 exemplarily for all ligands. 10 mM sodium acetate at pH 4.5 (#BR-1003-50, GE Healthcare) was selected as an immobilization buffer. As a running buffer, PBS was supplemented with 1.26 mM CaCl<sub>2</sub>, 0.493 mM MgCl<sub>2</sub> x 6H<sub>2</sub>O, 0.407 mM MgSO<sub>4</sub> x 7 H<sub>2</sub>O, and 5.33 mM KCl, since no HBSS was available in the facility. The system temperature was set to 37 °C. The chip flow cells were coated to equal units with recombinant murine CD206 constructs, or streptavidin as a negative control, using an amine coupling kit (#BR-1000-50, GE Healthcare). In order to determine binding affinities, 5-10 µl of graded doses of analyte, that is Japanin or OmCI, were injected at a flow rate of 10 µl/min. Japanin was diluted 9 times 1/2 from a 1 mg/ml stock; OmCI was diluted 1/4 and 1/8 from a 1 mg/ml stock.

## 2.8 Pull-down assays

### 2.8.1 Pull-down from Mutz-3-derived iDC and LC

A Dynabeads® co-immunoprecipitation kit (#143.21D, Invitrogen) was used to bind Japanin to magnetic beads for subsequent capture and isolation of receptors from cell lysate. About 5 mg beads were incubated overnight on a rotator at 37 °C in 250 µl Japanin at 1 mg/ml, or 250 µl PBS, as a negative control for unspecific binding. The next day, beads were washed and resuspended at 10 mg/ml. Success of the coating with Japanin was verified by a pull-out experiment with anti-his tag antibodies. For blocking of unspecific binding to uncoated areas of the beads, 30 µl bead suspension were washed with 250 µl

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3 % BSA in PBS containing 0.1 % Tween 20 (PBST) and 0.05 % NaAz. Subsequently, the beads were resuspended in 100 µl of a 1:1000 dilution of an anti-penta-his biotin conjugated antibody (#34440, Qiagen) in blocking solution and incubated on a rotator for 30 min at room temperature. Beads were washed twice with blocking solution, then resuspended in 50 µl 0.1 M glycine at pH 2.6 and incubated for 10 min at room temperature to separate any bound antibodies from the beads. 5 µl supernatant of Japanin coated beads and the PBS control were dot blotted neat, in 1:10, and in 1:100 dilutions on a nitrocellulose membrane and treated with peroxidase-conjugated streptavidin as described under 2.4.1. Only Japanin but not PBS-treated beads could pull-out anti-his tag antibodies, showing that the coating and blocking had been successful.

For the pull-down of Japanin-specific receptors, Mutz-3-derived iDC and LC were generated as described under 2.1.2. Around  $7 \times 10^7$  cells of each type were pelleted by centrifugation at 1200 rpm at 4 °C for 5 min and weighed. An extraction buffer was made up of diluted immunoprecipitation (IP) buffer from the kit and supplemented with 150 mM NaCl, 2 mM CaCl, 1 mM phenylmethanesulfonylfluoride (PMSF), and Halt™ protease inhibitor cocktail (#1862209, Thermo Scientific). About 0.4 g pelleted cells were resuspended in extraction buffer in a 1:9 ratio and incubated for 15 min on ice. The suspension was centrifuged at 4000 rpm or 2900 x g at 4 °C for 5 min and the supernatant was decanted into a fresh tube. Cell lysates of iDC and LC were each incubated with 150 µl Japanin-coated beads for 1 hour at 4 °C while constantly mixing on a rotator. Afterwards, the magnetic beads were gently washed four times with extraction buffer. Eventually the beads were resuspended in 60 µl elution buffer (EB) from the kit and incubated for 5 min at RT, meanwhile gently shaking the tubes by hand. The tubes were placed in a magnet and the bead supernatant was transferred into fresh tubes. 60 µl of each eluate were

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prepared for SDS-PAGE as described under 2.4, using thoroughly cleaned equipment and fresh reagents to minimize background signals in the following analysis. Two gels were prepared, one with 9  $\mu\text{l}$  of each sample for silver staining and one with the rest (split up between 3 wells) for mass spectrometric analysis. For mass spectrometry, the gel electrophoresis was stopped after 10 min and the lanes of iDC and LC samples were cut out separately and chopped into small cubes.

### 2.8.2 Pull-down from moDC after cross-linking

For the pull-down of Japanin-binding receptors from moDC after cross-linking,  $2.5\text{-}5 \times 10^7$  cells were thawed and resuspended in HBSS/FCS at  $0.5 \times 10^7/\text{ml}$  and split between two tubes. Either biotinylated Japanin or OmCI was added at 3  $\mu\text{g}/\text{ml}$  to the suspension and incubated for one hour on ice. Cells were washed three times, first in 50 ml HBSS/FCS, then in HBSS, and finally in PBS (pH 8). After these wash steps each cell pellet was resuspended in PBS (pH 8) to  $1 \times 10^7/\text{ml}$ , and bissulfosuccinimidyl suberate (BS<sup>3</sup>) (#21585, Thermo Scientific) was added to a final concentration of 1 mM. Cross-linking was allowed for 30 min at room temperature. To stop the reaction, Tris (pH 7.7) was added to a final concentration of 20 mM, plus another 13 ml cold PBS containing 20 mM Tris. After centrifugation at 1200 rpm and 4 °C for 5 min the supernatant was removed and the cell pellets were lysed for 15 min on ice in 600  $\mu\text{l}$  lysis buffer, consisting of 50 mM Tris pH 7.7, 150 mM NaCl, 2-5 mM EDTA, and 1 % Triton™ x-100, and also containing Halt™ protease inhibitor cocktail (#1862209, Thermo Scientific) and 1 mM PMSF. The lysates were centrifuged in a microfuge at 13000 rpm and 4 °C for 10 min. The supernatants were transferred into fresh tubes on ice and 40-50  $\mu\text{l}$  of each were put aside for SDS-PAGE and western blotting. Generally conducted as described under 2.4, but without DTT and an

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incubation for 10 min at 40 °C instead of 70 °C, about 20 µl of each sample alongside 20 ng biotinylated Japanin and OmCI were analysed. The remaining sample was mixed with 125 µl streptavidin beads (#11350D, Invitrogen), which had been washed four times with sterile PBS. After 1 hour incubation at 2-8 °C on a rotator, the beads were washed with 0.6-1 ml lysis buffer plus protease inhibitors, then with PBS containing 5 mM EDTA, then three times with PBS, finally resuspended in 60-100 µl, then frozen at -80 °C and delivered on dry ice to the Sir William Dunn School of Pathology at the University of Oxford for mass spectrometric analysis.

## 2.9 siRNA knock-down of CD206 in moDC

Monocytes were isolated as described under 2.2 and immediately transfected with siRNA by electroporation. A pool of four different siRNAs (ON-TARGETplus SMARTpool, #L-011730-00, Thermo Scientific Dharmacon) was used to target the human CD206 gene, and a non-targeting siRNA pool (ON-TARGETplus Non-targeting Pool, #D-001810-10-05, Thermo Scientific Dharmacon) was used as a negative control. 100 µM stocks of siRNA were made up following the basic siRNA resuspension protocol of the manufacturer, using 5 x siRNA universal buffer (#1182-1924, Fisher Scientific) and molecular biology grade water (#1049-0025, Fisher Scientific). Monocytes were concentrated to  $2 \times 10^7$ /ml in OptiMEM I (#31985-062, Life Technologies) and 100 µl suspension per condition were mixed with 3 µl siRNA in a 0.2 cm cuvette for electroporation (#165-2086, Bio-Rad). Using the Bio-Rad Gene Pulser® II electroporation system, monocytes were transfected with siRNA at 250 V, 150 µF, and  $\infty$  resistance. Immediately after electroporation, GM-CSF- and IL-4-supplemented complete RPMI was added to the cuvettes and monocytes were transferred into 12-well plates at  $1.3 \times 10^6$ /ml in 1.5 ml per well and cultured at 37 °C and 5 % CO<sub>2</sub> for differentiation into moDC (see 2.2).

## **2.10 Generation of DC and macrophages from mouse bone marrow**

Frozen bone marrow of female C57BL6 CD206 knock-out and wild type mice was received from Luisa Martinez-Pomares at the University of Nottingham.  $10^6$  cells were seeded in a non-treated 24-well plate (#3738, Costar) in 1 ml medium per well and incubated with recombinant murine M-CSF (#315-02, PeproTech) at 100 ng/ml for differentiation of macrophages. DC differentiation was induced with recombinant murine GM-CSF (#315-03, PeproTech) at 20 ng/ml. After 3 days of culture, cytokines were refreshed by adding 1 ml medium supplemented with M-CSF at 100 ng/ml or GM-CSF at 20 ng/ml. Macrophages and DC were harvested after 7 days of culture by pipetting the cells several times up and down and flushing the wells with PBS/EDTA (#BE02-017F, Lonza BioWhittaker®) twice.

## **2.11 Enhancement of the binding of Japanin to THP-1**

### **2.11.1 Chemical cross-linking**

THP-1 were cultured as described under 2.1 and a Japanin binding assay was performed as described under 2.7.1, only with the alteration that the cells were not washed in HBSS/FCS after the incubation with fluorescently labelled Japanin or OmCI; instead, chemical cross-linkers were added. Cells were incubated with Japanin or OmCI in the presence of either 1 % Formaldehyde (#28906, Thermo Scientific), 1 mM BS<sup>3</sup> (#21585, Thermo Scientific), or 1 mM DSS (#21658, Thermo Scientific) for 15 min on ice. Thereafter the cells were washed twice before analysis by flow cytometry.

### 2.11.2 Antibody conjugation

Generally, a Japanin binding assay was conducted with THP-1 cells as described under 2.7.1, except that all dilutions were made in PBS/EDTA plus 5 % mouse serum. In order to link multiple Japanin molecules, 10 µl of monoclonal mouse anti-his tag biotin-conjugated antibodies (#ab106261, abcam) were mixed with 400 µl diluted fluorescently labelled Japanin and incubated with the cells for 1 hour on ice. OmCI was treated in the same way as a negative control. The binding of the antibody was detected by FITC-conjugated streptavidin. FITC-conjugated mouse IgG (#015-090-003, Jackson Immuno Research) was used as an isotype control.

### 2.12 IL-10R-binding assays

We attempted to detect the binding of the interleukin-10 receptor (IL-10R) to immobilised Japanin and IL-10 in the manner established by Naiyer et al.; which is immobilising IL-10R ligands on a microtitre plate, then adding recombinant IL-10R and detect them by anti-IL-10R antibodies <sup>294</sup>. However, no binding could be detected by this method in our lab, possibly due to insufficient protein quantities. Therefore, the IL-10R binding experiment with Japanin was carried out in analogy to the CD206 plate binding studies described under 2.7.2. Briefly, 4 wells of a 96-well plate were each coated with 100 µl of recombinant human IL-10R $\alpha$  (#274-R1-050/CF, R&D Systems) at 1 µg/ml in PBS at 2-8 °C overnight. The plate was washed, and blocked with 300 µl per well of 3 % BSA in PBS for 1 hour at room temperature. After another wash, either 100 µl recombinant human IL-10 (#200-10, PeproTech) at 4 µg/ml or biotinylated Japanin or OmCI at 10 µg/ml, or blocking solution were added. 200 µl blocking solution were added additionally to each well and it was incubated for 2 hours at room temperature. 100 µl anti-IL-10 antibody (#MAB2171, R&D Systems) at 1 µg/ml in blocking solution were added and incubated for 1

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hour to detect the binding of IL-10. 100 µl of a 1:100 dilution of a HRP-conjugated anti-mouse secondary antibody (#P0447, Dako) was added to visualise the binding of the anti-IL-10 antibody; the secondary antibody was also added to the well that had been treated as a negative control with blocking solution only. To visualise the binding of biotinylated Japanin or OmCI, the plate was incubated in peroxidase-conjugated streptavidin for 1 hour at room temperature, then washed and incubated in ABTS for 10 min at 37 °C. The absorption was measured at 405 nm and 690 nm.

## 3 Results

### 3.1 Modulatory functions of Japanin

The aim of this chapter was to further investigate the immunomodulatory activity of Japanin; most of its DC-modulatory functions have already been published by us <sup>290</sup>. Firstly, the production and purification procedure of recombinant Japanin will be illustrated in more detail. Thereafter the activity of Japanin on the maturation and differentiation of moDC will be demonstrated in the presence of different stimuli and by different measures. Moreover, in addition to what has already been published, results of mixed leucocyte reactions (MLRs) with moDC and T cells will provide novel insights into extended effects of Japanin on T cells.

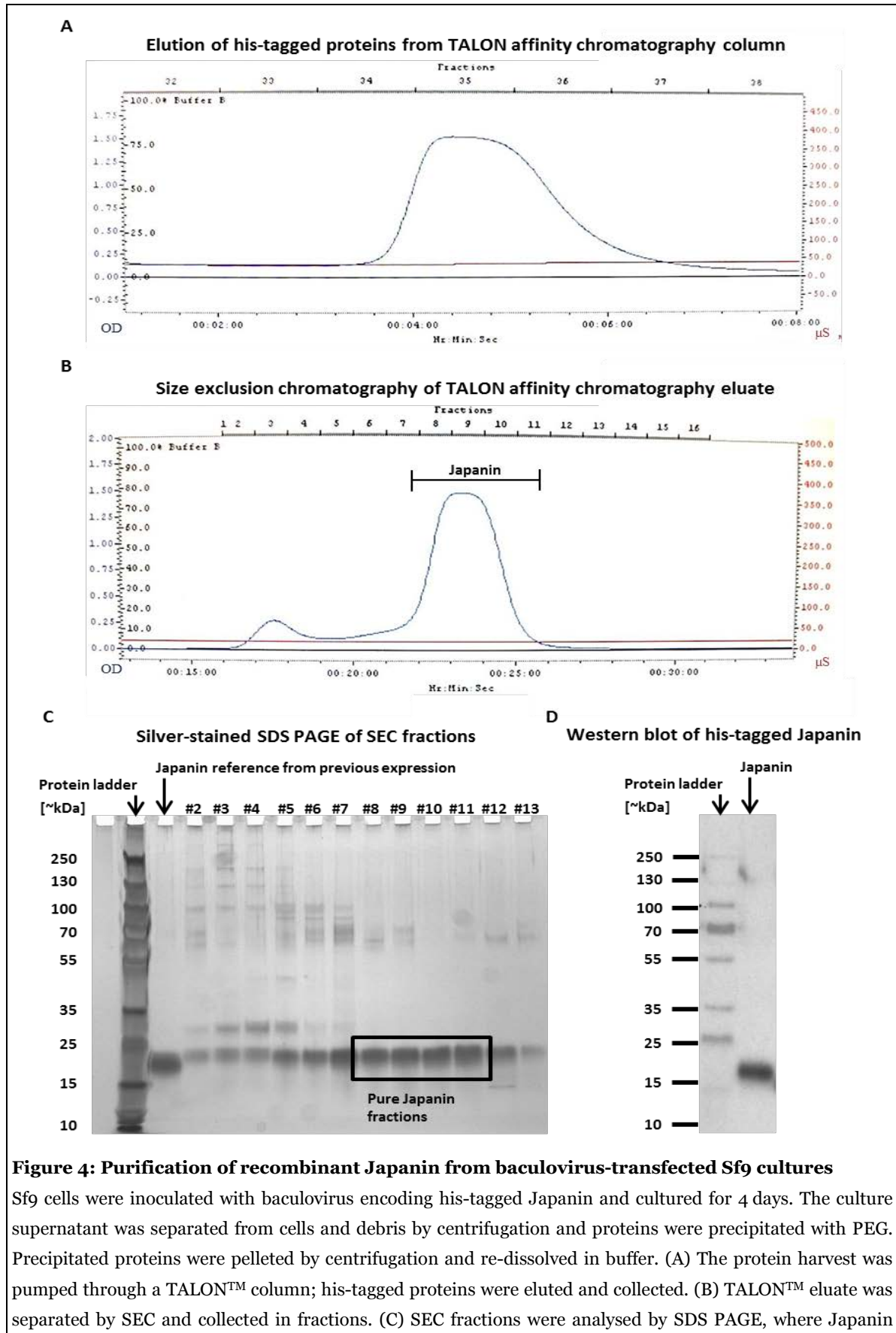
#### 3.1.1 Expression and purification of recombinant Japanin

Previous work in our lab originally identified the DC-modulatory activity of Japanin in SGE of *R. appendiculatus* ticks <sup>290</sup>. SGE showed the capacity to modulate DC maturation in response to bacterial LPS, as measured by inhibited expression of the co-stimulatory molecule and maturation marker CD86. The modulation was shown to be protein-dependent by treatment of SGE with Proteinase K, which abolished the DC-modulatory activity. The active SGE fraction was isolated by multiple rounds of chromatography and an N-terminal protein sequence was generated which allowed the design of degenerate primers for cloning of a cDNA. The Japanin sequence was inserted into vectors for mammalian and bacterial expression as well as a recombinant baculovirus for expression in insect cells. Japanin was harvested from supernatants of transfected mammalian cells; however, the yields were very low, since the transfections were only transient. No Japanin could be obtained from bacterial cultures, but insect cells transfected with baculovirus

secreted the recombinant protein. The DC-modulatory activity of recombinant Japanin was confirmed by the ability of the mammalian and insect cell transfection supernatants to prevent CD86 upregulation by moDC upon stimulation with LPS, as seen in SGE.

In order to further investigate the functions of Japanin within this study, the protein was produced using the baculovirus expression system. A great advantage of this expression method compared to transient expression in mammalian cells was, that higher yields could be obtained. This allowed subsequent purification of Japanin from the transfection supernatant and the production of concentrated stocks for the determination of an effective working concentration of Japanin on moDC. Briefly, the insect cell line Sf9 was transfected with recombinant baculovirus containing the cDNA encoding Japanin plus a polyhistidine tag. Culture supernatants were harvested and proteins were precipitated with PEG. The precipitate was then re-dissolved in buffer and applied to an immobilized metal affinity chromatography resin charged with cobalt (TALON™), which binds his-tagged proteins (Figure 4 A). The eluate fraction was then further separated by SEC (Figure 4 B). Peak fractions were collected and analysed by SDS PAGE and subsequent silver staining (Figure 4 C), where Japanin appeared as a band of about 20 kDa, as was confirmed by western blots probed with antibodies against the polyhistidine tag (Figure 4 D). The fractions containing the purest Japanin at sufficient concentrations were pooled and sterile-filtered for the following studies. Typically, 1 litre of transfected Sf9 suspension yielded 1 mg purified Japanin protein. Successive dilutions of the protein were titrated on LPS-stimulated moDC from different donors. The expression of CD86 decreased with increasing Japanin concentration, showing that its effect was dose-dependent. 500 ng/ml was established as an effective working concentration that could significantly reduce the expression of CD86; a representative titration is shown in Figure 5 C.

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**Figure 4: Purification of recombinant Japanin from baculovirus-transfected Sf9 cultures**

Sf9 cells were inoculated with baculovirus encoding his-tagged Japanin and cultured for 4 days. The culture supernatant was separated from cells and debris by centrifugation and proteins were precipitated with PEG. Precipitated proteins were pelleted by centrifugation and re-dissolved in buffer. (A) The protein harvest was pumped through a TALON™ column; his-tagged proteins were eluted and collected. (B) TALON™ eluate was separated by SEC and collected in fractions. (C) SEC fractions were analysed by SDS PAGE, where Japanin

fractions were identified by a molecular weight of about 20 kDa. (D) Pure Japanin fractions were pooled and identified by anti-his tag antibodies via western blot. Representative experiments are shown.

### 3.1.2 Japanin alters DC maturation towards a tolerogenic phenotype

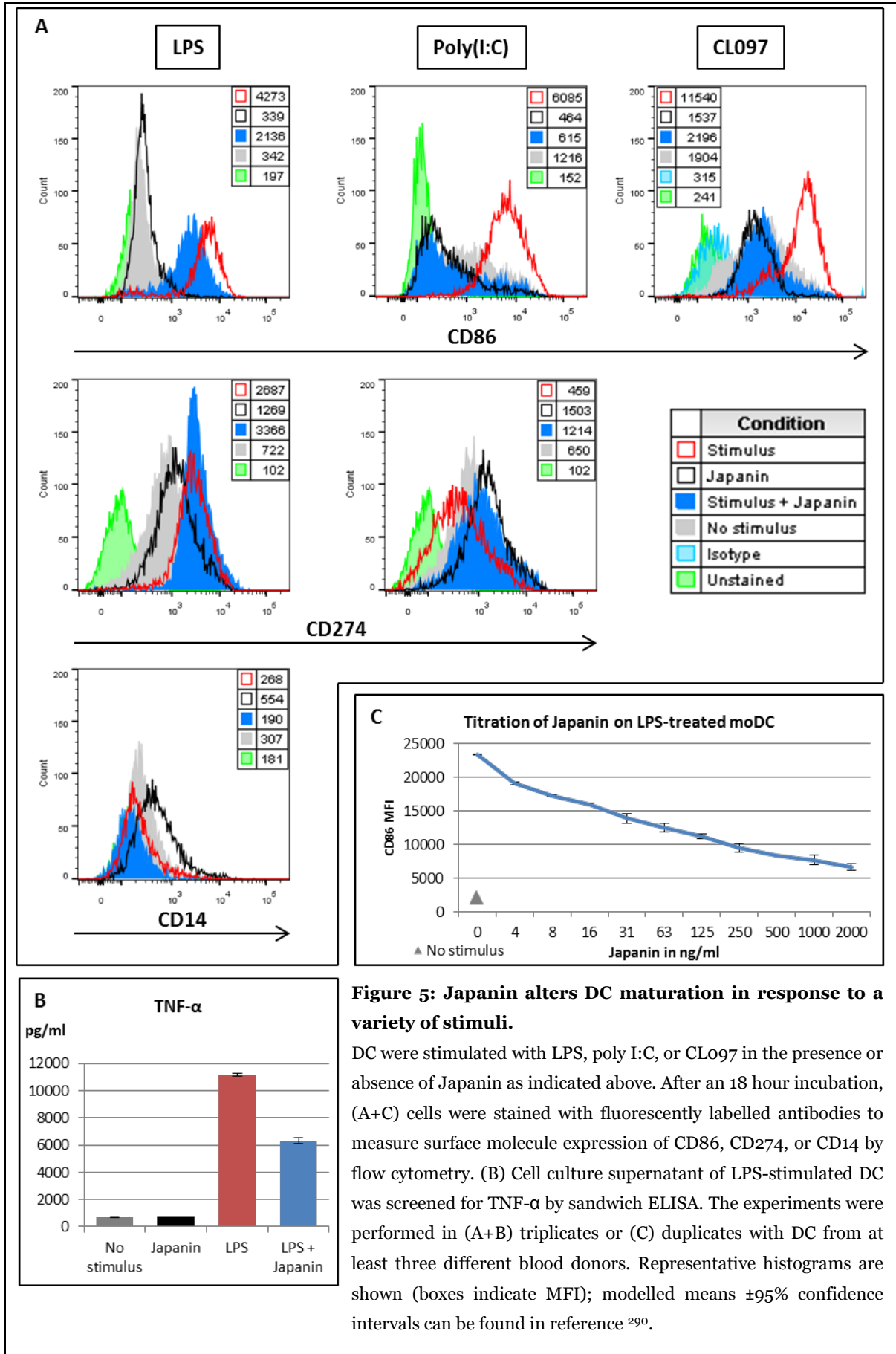
As professional APCs, DC possess the capacity to initiate and regulate adaptive immune responses. Once they have matured upon sensing pathogens or self-associated danger signals, DC present antigens on MHC molecules and express co-stimulatory molecules, such as CD86, or co-inhibitory molecules, such as CD274, to T cells. Mature DC can moreover stimulate or suppress immune responses by secretion of pro- or anti-inflammatory cytokines. Whether DC show an immunogenic or tolerogenic phenotype depends on their microenvironment which, *in vivo*, is shaped by cytokines secreted from other immune cells or pathogenic components, and *in vitro* by addition of recombinant mediators or chemicals.

Since Japanin was originally derived from tick SGE, we hypothesised that it induced not only the downregulation of CD86, but an overall tolerogenic DC phenotype that would allow ticks to feed for several days without being rejected by the host immune system. In order to test this hypothesis, moDC were stimulated with LPS, in the presence or absence of Japanin, and screened for expression of maturation markers and cytokines. It was observed that in addition to its diminishing effects on the co-stimulatory molecule CD86 (Figure 5 A), Japanin also reduced secretion of the pro-inflammatory cytokine TNF- $\alpha$  (Figure 5 B). Furthermore, the DC maturation marker CD83 as well as the cytokines, G-CSF, IL-1, IL-6, IL-12, and CXCL10 were downregulated by moDC upon treatment with Japanin, as demonstrated by others <sup>290</sup>. At the same time, Japanin upregulated co-inhibitory molecule CD274 (Figure 5 A) and increased release of the anti-inflammatory cytokine IL-10, as shown by others <sup>290</sup>. Japanin also demonstrated the ability to modulate

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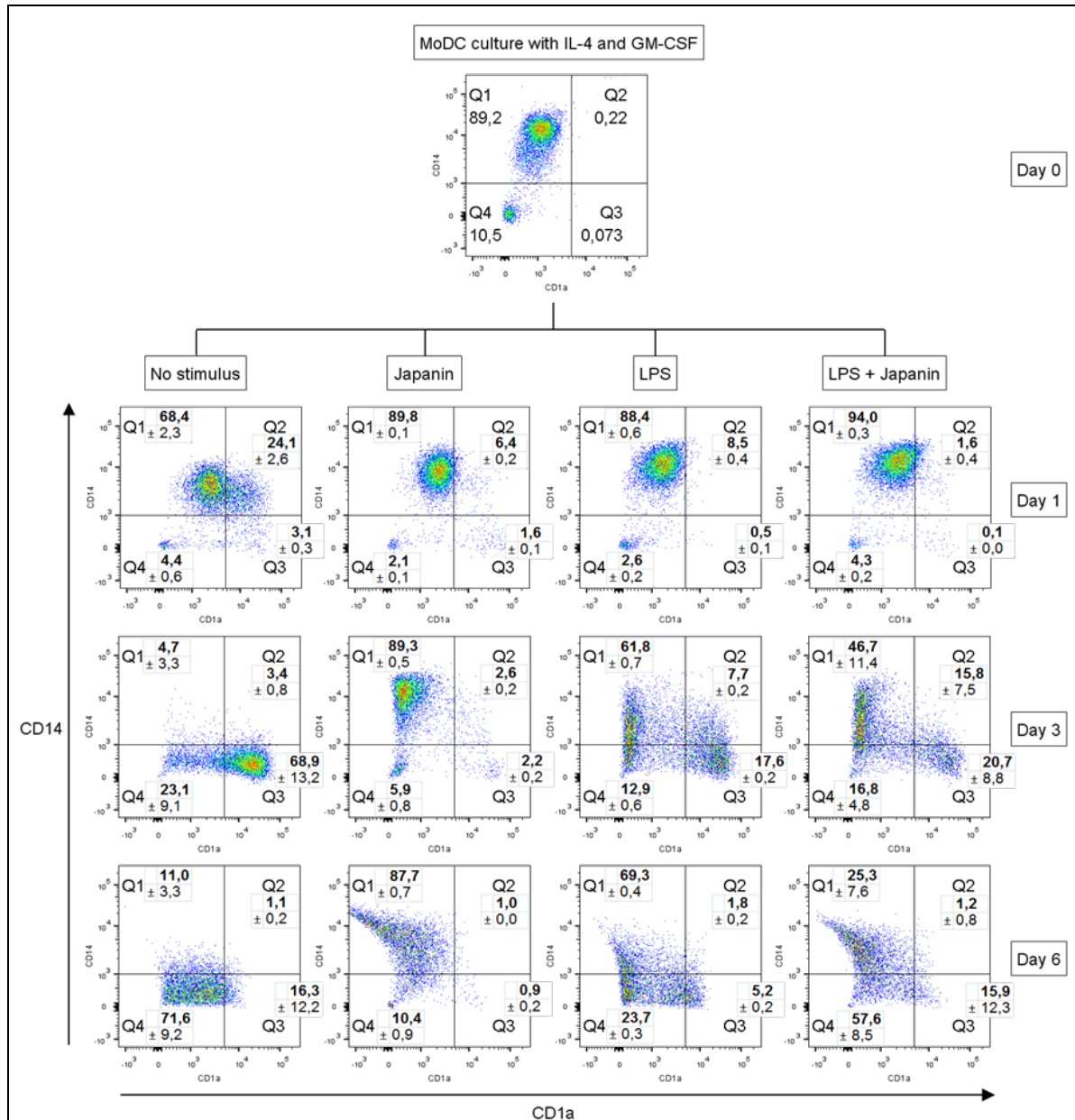
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moDC in response to stimuli other than the TLR4 agonist LPS, targeting different TLRs and cytokine receptors. Measured by CD86 expression, Japanin altered DC maturation upon stimulation by TLR3 agonist poly(I:C) and TLR7/8 agonist CL097 (Figure 5 A), as well as the cytokines IFN- $\alpha$  and IFN- $\gamma$ , while TNF- $\alpha$ -induced CD86 was not significantly decreased by Japanin, as shown by others <sup>290</sup>.



### 3.1.3 Japanin prevents moDC differentiation

Throughout this study, human moDC were generated *in vitro* from monocytes cultivated in an IL-4- and GM-CSF-supplemented differentiation medium. *In vivo*, DC can originate directly from bone marrow precursors, but also monocytes can be recruited from the blood into tissues such as the skin, where they differentiate into moDC in support or replacement for conventional myeloid DC populations. Having observed the modulatory functions of Japanin on moDC maturation, we hypothesised that this might be part of a tick mechanism to evade host immunity. Hence, we reasoned that Japanin may also modulate the differentiation of monocytes into moDC. In order to test whether Japanin influenced the development of moDC from monocytes, moDC differentiation cultures were set up in the presence of Japanin and cells were screened for the expression of monocyte- and moDC-specific markers during the culture. In addition to a reference culture without Japanin, moDC differentiation cultures were also set up in the presence of LPS, with and without Japanin, as LPS is known to hinder the development of monocytes into moDC<sup>34</sup>. We found that Japanin impaired the downregulation of the monocyte marker CD14 as well as the upregulation of the moDC marker CD1a, suggesting that it arrested moDC differentiation (Figure 6). Notably, Japanin was a more potent inhibitor of moDC differentiation than LPS, which caused retention of the CD14<sup>+</sup> CD1a<sup>-</sup> monocyte phenotype in only about half of the cells. Japanin-treated moDC cultures on the other hand, maintained this monocytic phenotype for 6 days. Moreover, Japanin caused upregulation of CD14 on immature, but not LPS-stimulated, moDC, suggesting that Japanin might reverse the differentiation of moDC in the absence of additional stimuli (Figure 5 A).



**Figure 6: Japanin prevents moDC differentiation.**

Monocytes were cultured in the presence of IL-4 and GM-CSF only, or with additional stimulation by Japanin and/or LPS. Cytokines were refreshed on day 3. The expression of CD1a and CD14 was measured by flow cytometry before the addition of cytokines (day 0) and on days 1, 3, and 6 of the culture, in order to assess differentiation into moDC. Compensation controls (unstained, CD14-PE, CD1a-FITC) were set up and measured on each staining day, yet the photomultiplier voltage settings were not changed after day 0. The experiment was set up in triplicates on days 1, 3, and 6; mean values  $\pm$  standard error are indicated in each quartile; representative dot plots are shown.

#### 3.1.4 Japanin modulates T cell functions in a DC-dependent manner

The capacity to prime naïve T cells makes DC the most potent APCs, as they enable T cells to proliferate, differentiate into effector subsets and respond to antigens presented by other cells. The TCR of cytotoxic CD8<sup>+</sup> T cells may recognise peptides from antigens presented on MHC class I molecules by almost all cells of the body. Cytotoxic T (Tc) cells are then able to induce death of cells they identify to be damaged or infected. The TCR of CD4<sup>+</sup> T helper cells on the other hand recognises peptides presented on MHC class II molecules by professional APCs, such as DC, B cells, or macrophages. T helper cells specialise in the secretion of cytokines to support cellular or humoral immunity. However, cytotoxic T cells can also produce subset-specific cytokines. By providing a combination of signals during the priming process, DC can influence the differentiation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells into polarised effector subsets, which secrete characteristic cytokines.

Japanin altered the expression of surface molecules and cytokines in moDC that are crucial for the interactions with T cells. The activity of Japanin on moDC included reduced expression of co-stimulatory molecules and decreased secretion of pro-inflammatory cytokines, while up-regulating the expression of CD274 as well as the production of IL-10. We therefore hypothesised that the presence of Japanin in an MLR would prevent moDC from promoting T cell proliferation or polarisation. In order to test whether Japanin-treated moDC were unable to activate T cells, proliferation and cytokine production of T cells were measured in allogeneic MLRs. In these *in vitro* assays, moDC from one donor were co-cultured with T cells from another, which may be activated upon recognition of antigens presented by allogeneic moDC. *In vivo*, the phenomenon of alloreactivity is observed as graft rejection upon organ transplantation. Hence, the inhibition of T cell activity in an MLR could potentially be useful for medical applications. From an

experimental point of view, the alloreactive MLR has the advantage that no previous immunisation is needed to generate T cell proliferation. However, if desired, stimulation of DC with PAMP, such as LPS, can enhance their capacity to promote T cell proliferation and moreover, can induce production of T cell-polarising cytokines.

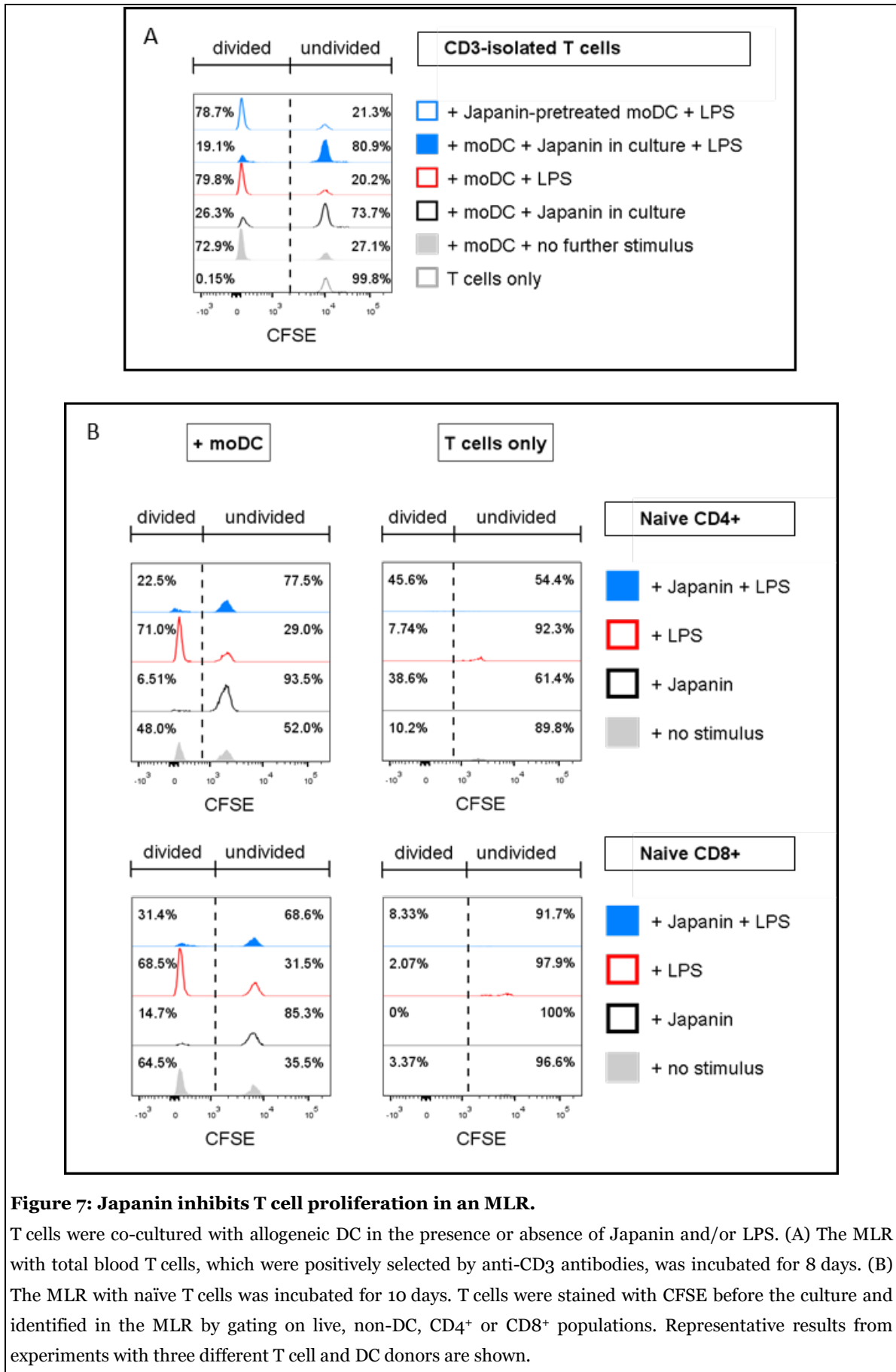
### *Japanin inhibits T cell proliferation in an MLR*

In an initial allogeneic MLR experiment, T cells from one donor were isolated by their expression of CD3 and incubated with moDC generated from another donor. T cell proliferation was measured by staining with CFSE, a fluorescent cell dye that enables monitoring of proliferation, because its fluorescence halves each time a stained cell divides<sup>295</sup>. We noticed that T cells appeared essentially in only two peaks: CFSE<sup>high</sup> cells and CFSE<sup>low</sup> cells. Although this limited interpretation of the exact number of cell divisions, CFSE<sup>high</sup> cells had most likely not divided while CFSE<sup>low</sup> cells had presumably divided seven times or more<sup>296–299</sup>. T cells proliferated in the presence of DC, as opposed to when cultured without DC (Figure 7 A). Addition of LPS to the MLR increased the division rate of T cells. The presence of Japanin however, inhibited the proliferation of T cells, even when LPS was present in the culture. Interestingly, this was only the case when Japanin was continuously present in the MLR, but not when DC were only pre-treated with Japanin and washed before the co-culture with T cells, indicating that the effects of Japanin on moDC are reversible. On the other hand, this observation could also suggest that Japanin had direct effects on T cells and therefore needed to be present in the MLR. This suggestion was however disproved by previous experiments in our lab in which Japanin could not inhibit T cell proliferation when induced by beads coated with anti-CD3 and anti-CD28 antibodies instead of DC (personal communication, Stephen Preston).

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DC are able to activate both naïve and memory T cells, but CD3-based isolation of T cells did not allow discrimination between the two populations. Therefore in the next set of MLR experiments, naïve T cells were isolated specifically in order to investigate the activity of Japanin on primary responses. It was found that spontaneous allogeneic as well as LPS-amplified proliferation of naïve CD4<sup>+</sup> and CD8<sup>+</sup> T cells could be inhibited by Japanin in the MLR (Figure 7 B). The division rate of T cells cultured without DC was expectedly very low due to missing priming stimuli. Hence, it was impossible to draw conclusions from this experiment as to whether there were any potential DC-independent effects of Japanin that might impair the proliferation of naïve T cells directly. Yet these results confirmed that Japanin can inhibit the proliferation of naïve T cells in a DC-dependent fashion.



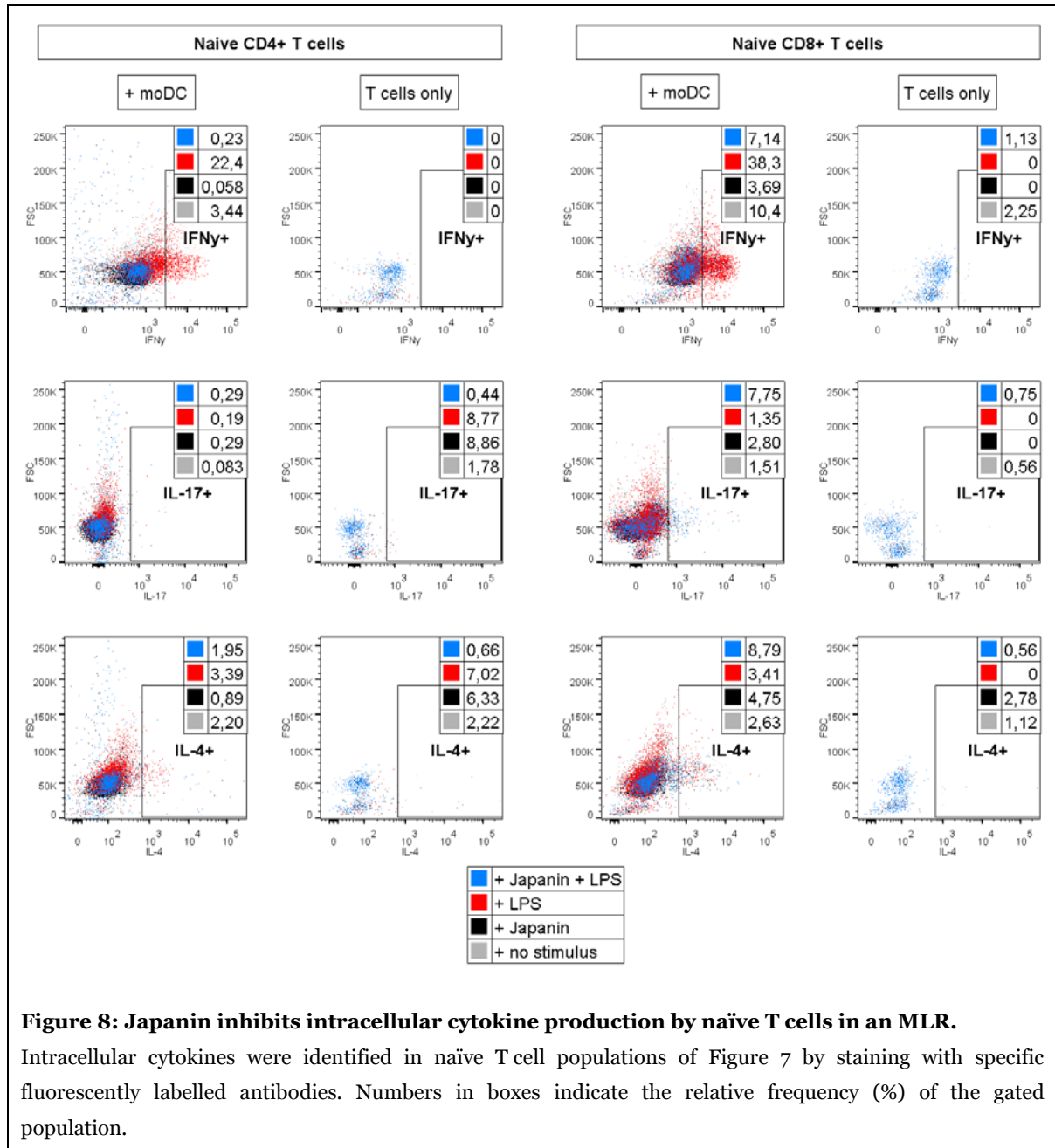
**Figure 7: Japanin inhibits T cell proliferation in an MLR.**

T cells were co-cultured with allogeneic DC in the presence or absence of Japanin and/or LPS. (A) The MLR with total blood T cells, which were positively selected by anti-CD3 antibodies, was incubated for 8 days. (B) The MLR with naïve T cells was incubated for 10 days. T cells were stained with CFSE before the culture and identified in the MLR by gating on live, non-DC, CD4<sup>+</sup> or CD8<sup>+</sup> populations. Representative results from experiments with three different T cell and DC donors are shown.

*Japanin decreases intracellular cytokine expression in T cells*

Having found that Japanin altered the cytokine secretion of DC in response to LPS, we hypothesised that this could modulate their capacity to induce T cell polarisation. LPS alone was shown to induce INF- $\gamma$  and IL-12 in moDC, which may drive the polarisation of Th1 or Tc1 cells, as well as IL-1 $\beta$  and IL-6, which may promote the generation of Th17 or Tc17 cells. The presence of Japanin significantly reduced the secretion of these cytokines by moDC<sup>290</sup>. Therefore we would expect that Japanin prevented the differentiation of Th1/Tc1 and Th17/Tc17 cells in an MLR; and possibly, this might favour of the polarisation of Th2/Tc2 cells. Hence the intracellular cytokine expression was measured in naïve CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the MLR with allogeneic DC in the presence of Japanin, LPS, both, or no additional stimulus. As a control for the DC-specificity in the induction of cytokine expression in T cells, each condition was also set up without moDC. We found that only in the presence of both DC and LPS, distinct populations of CD4<sup>+</sup> and CD8<sup>+</sup> T cells showed elevated levels of Th1/Tc1-characteristic IFN- $\gamma$ , while naïve T cells incubated with LPS or DC alone did not show increased expression of IFN- $\gamma$  (Figure 8). This suggested that the induction of IFN- $\gamma$  in T cells was mediated through the stimulation of DC by LPS. Addition of Japanin to the MLR prevented the generation of IFN- $\gamma$  production by T cells, suggesting that Japanin inhibited the effects of LPS. On the other hand, an increase in intracellular IL-17 and IL-4 was only observed in few randomly distributed T cells, suggesting that neither the presence of LPS nor Japanin in the MLR induced Th17/Tc17 or Th2/Tc2 cells.

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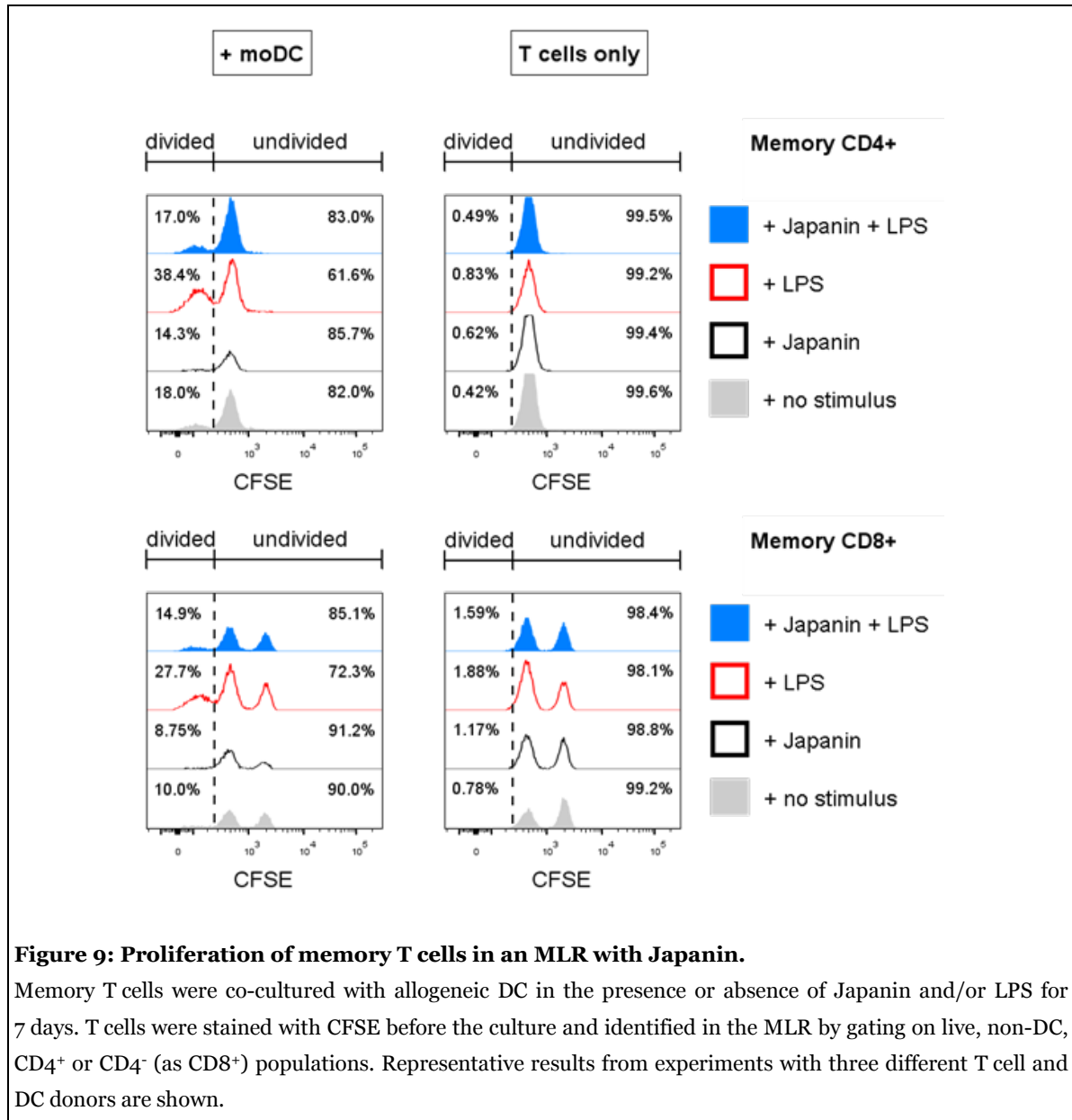


Overall, the MLR results may suggest that Japanin arrests naïve T cells by preventing their priming through DC. Since Japanin demonstrated ability to inhibit T cell proliferation in the MLR, it could be argued that the reduced IFN- $\gamma$  expression might be a secondary effect resulting from impaired growth, rather than specifically blocked IFN- $\gamma$  production. Moreover, it would have been interesting to investigate whether the expression of IL-10 was induced in naïve T cells in the presence of allogeneic DC and Japanin, as this could

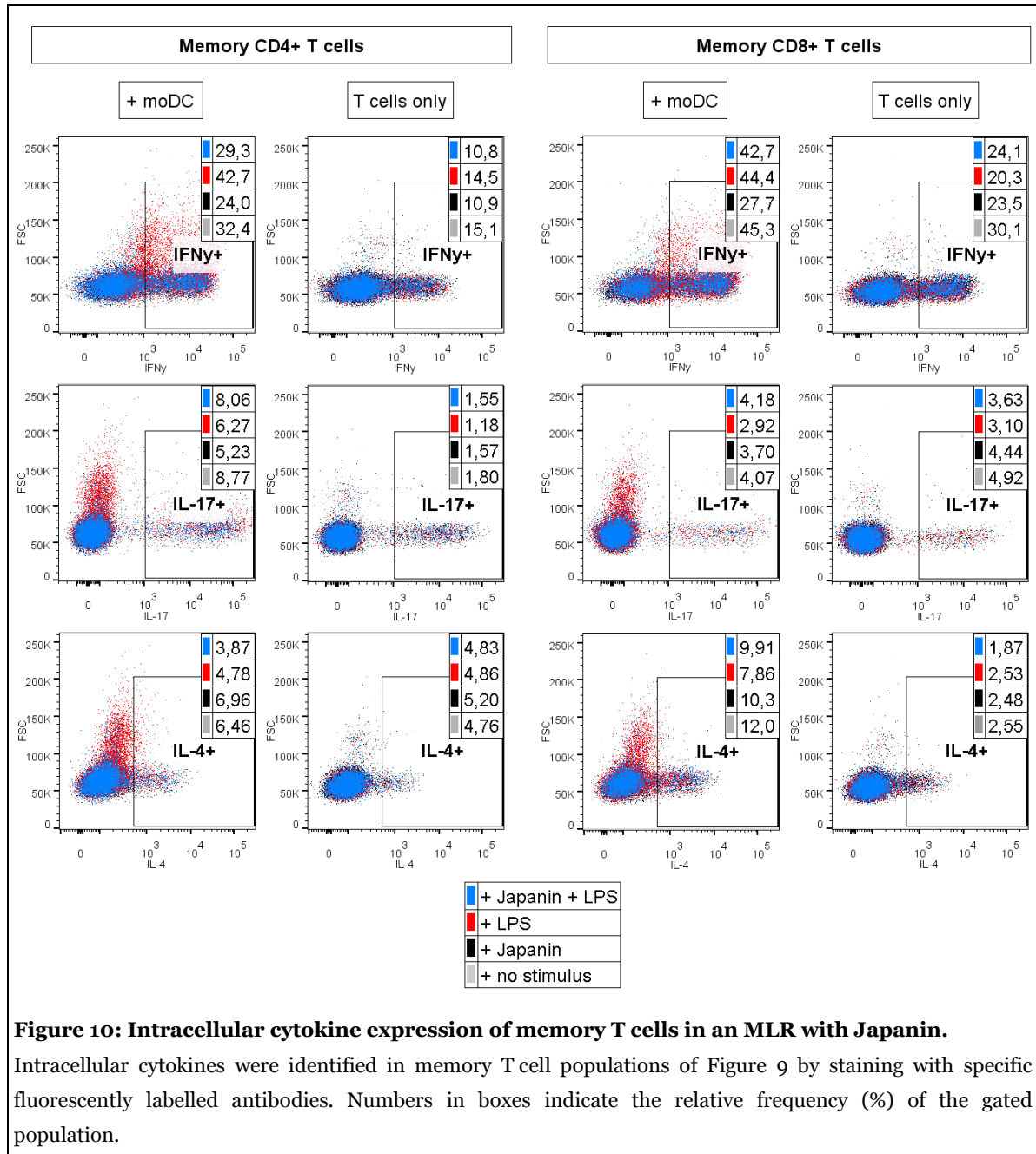
have indicated that Japanin-modified moDC may promote the polarisation of Tr1 cells. Unfortunately, antibodies against IL-10 were not included in our MLRs, since these assays were set up in context of adjuvant studies, for which IL-10 was irrelevant.

Similar MLRs were performed with memory T cells. However, there was essentially no T cell proliferation observable on days 2 and 5 of culture in three independent experiments with DC and memory T cells from different donors. Hence, it was not possible to measure any significant Japanin-specific effects on memory T cell responses. Only in one experiment some memory T cells had proliferated and produced IFN- $\gamma$  in the presence of allogeneic moDC and LPS by day 7 of the MLR culture (Figure 9, Figure 10). Unfortunately, CD8<sup>+</sup> T cells were insufficiently stained in this experiment so that this population could only be determined by gating on CD4-negative T cells. This made it difficult to distinguish accurately between CD4<sup>+</sup> and CD8<sup>+</sup> memory T cells and may explain why the CFSE staining of the supposed memory CD8<sup>+</sup> T cell population shows an extraneous peak. Proliferation was not observed in the presence of Japanin, which could suggest that Japanin might inhibit the ability of moDC to activate memory T cells; in addition to the limited ability to prime naïve T cells. However, this result was not reproducible due to overall limited responses by memory T cells. It is explainable that memory T cells from healthy individuals would not respond to allogeneic DC alone, because they had never encountered alloantigens before <sup>300</sup>. Yet they should respond to LPS-presenting allogeneic DC, if the HLA-type matched <sup>301</sup>. Since we do not know the HLA-type of the blood donors, it might be possible that there was an HLA-match in only one of the three experiments. Given that the T cells did not proliferate until day 7, it might also be possible that the isolation procedure of memory T cells did not eliminate all naïve

T cells, which should have bound to negative selection beads coated with anti-CD45RA-antibodies.



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### 3.1.5 Summary of chapter 3.1

This chapter has illustrated the production and purification of recombinant Japanin from baculovirus-transfected Sf9 cultures. The purified protein was then used to demonstrate the modulatory functions of Japanin on DC maturation, as shown by decreasing the expression of CD86 and TNF- $\alpha$ , whilst increasing the expression of CD274, overall suggesting the induction of a tolerogenic DC phenotype. Japanin was also found to impair differentiation of monocytes into moDC, as indicated by preserved expression of CD14 and reduced expression of CD1a when monocytes were cultured in the presence of GM-CSF, IL-4 and Japanin. Furthermore, the modulatory activity of Japanin extended to T cells in a DC-dependent manner, as was concluded from diminished T cell proliferation and cytokine expression in allogeneic MLRs. Considering the immunoregulatory significance, understanding the mode of action of Japanin could have potential value for therapeutic application, for example in the prevention or treatment of transplant rejection or autoimmune diseases.

## 3.2 Identification of CD206 as a Japanin-binding receptor

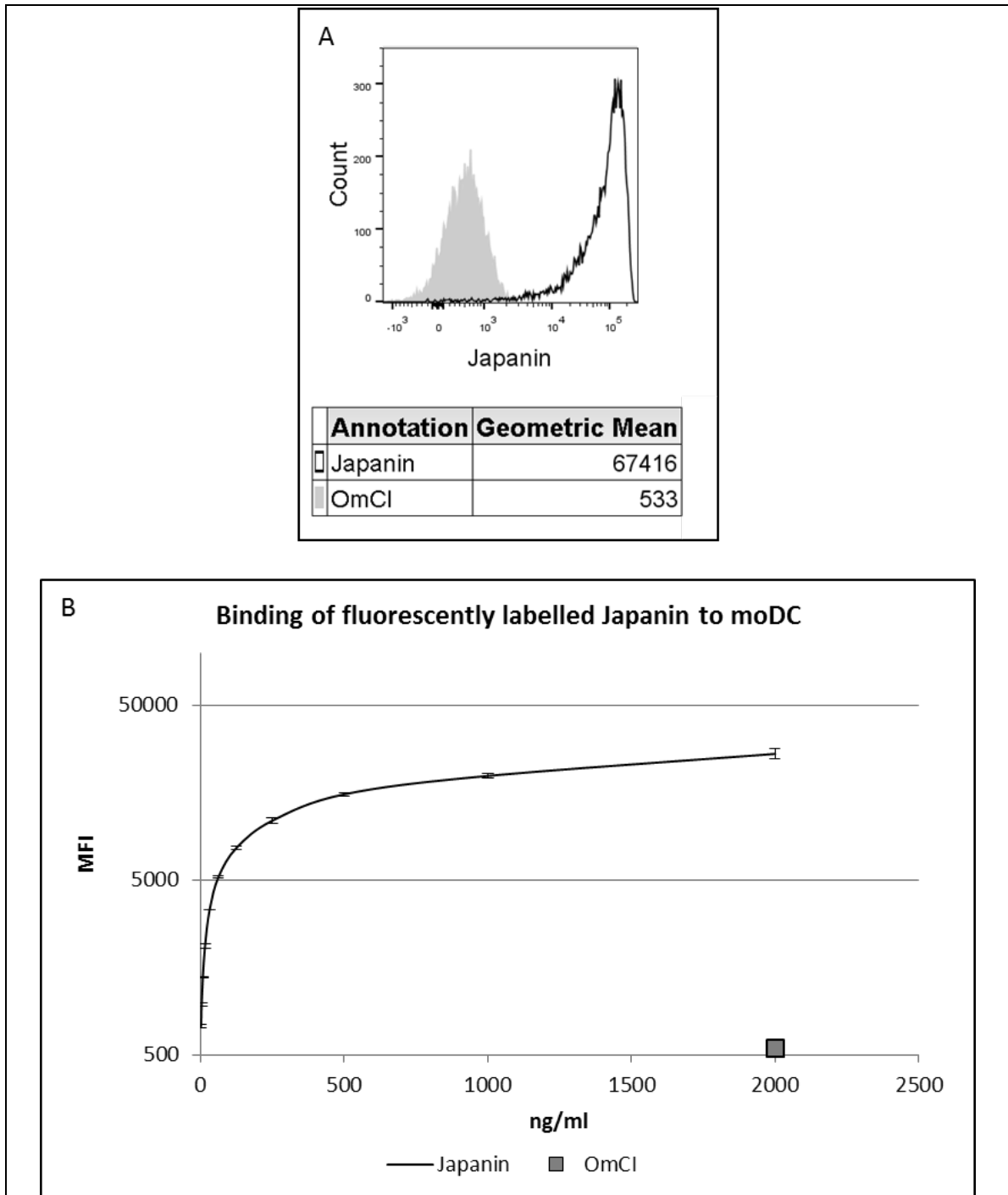
In this chapter, we aim to identify Japanin-specific receptors on DC. Therefore, we will initially measure the binding of labelled Japanin to different leucocyte populations in human blood, in order to determine whether Japanin binds specifically to DC. Japanin-binding cells will be used to isolate Japanin-specific receptors in pull-down assays. The specificity of the identified receptors for Japanin will then be verified by binding studies with recombinant receptors, as well as by specific knock-down of putative Japanin-binding receptors in Japanin-binding cells, which should abolish the binding.

### 3.2.1 Identification of Japanin binding cells

Opposed to other tick SGE-derived immunomodulators that may have multiple functions on different immune cells, Japanin appeared to be a DC-specific modulator, since it had no direct activity on T cells. Moreover, previous work by others had shown that differentiating moDC increasingly bound Japanin <sup>290</sup>. These findings suggested the presence of Japanin-specific receptors on DC, which were upregulated during differentiation from monocytes. Hence we set out to investigate whether Japanin triggered receptors that were exclusively expressed by DC, as these would potentially mediate the modulatory activity of Japanin. In order to assess the specificity of Japanin for DC, we screened for the binding of Japanin to different leucocyte populations from human blood, as well as moDC, and a number of monocytic or DC-like cell lines. Recombinant Japanin protein was directly labelled with a fluorochrome that enabled subsequent analysis of its binding to cells by flow cytometry. As a negative control, OmCI was labelled in the same manner, as it is also a recombinant tick SGE-derived lipocalin, but has no known functions on DC. All binding assays with cells were set up on ice, to prevent or reduce non-specific

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uptake of Japanin or OmCI by phagocytosis or macropinocytosis. Flow cytometric analysis confirmed that fluorescently labelled Japanin bound to moDC in a dose dependent manner, while OmCI did not (Figure 11).



**Figure 11: Japanin binds to moDC.**

MoDC were incubated with fluorescently labelled Japanin or OmCI for 1 hour on ice, washed, and analysed by flow cytometry. (A) Japanin or OmCI were applied at 1  $\mu$ g/ml. A representative result from >10 experiments

with different donors is shown. (B) Successive dilutions were performed in duplicates; graph shows the arithmetic mean; error bars indicate the standard error. A representative curve from experiments with >2 different donors is shown.

*In human blood, Japanin specifically binds DC or -precursors*

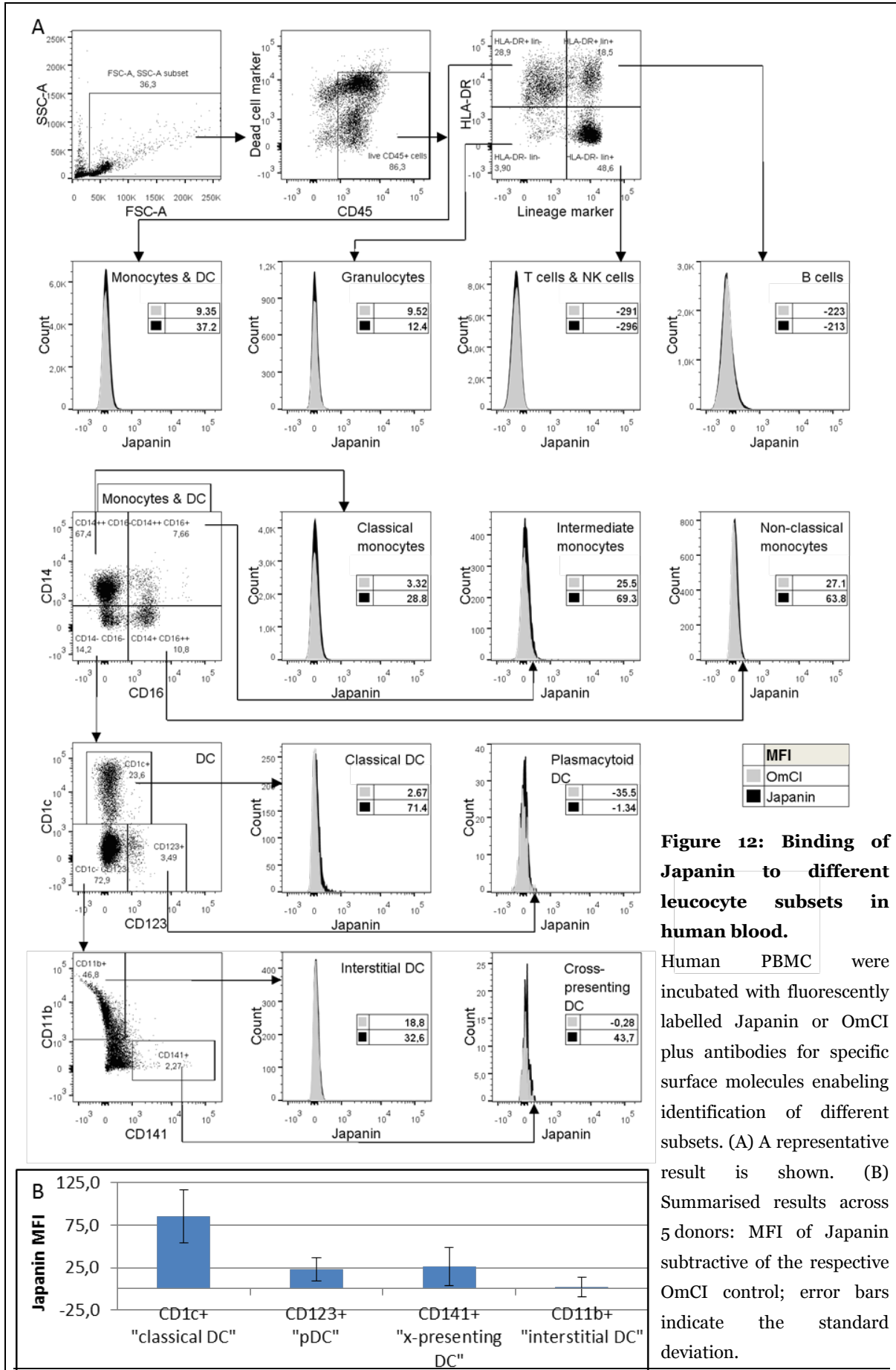
In order to investigate whether Japanin-binding receptors were exclusively expressed by DC, human PBMC were isolated by density gradient centrifugation and stained with Japanin and a panel of antibodies which allowed gating on different subsets (Figure 12). CD45 was used to discriminate between haematopoietic cells of interest and any residual erythrocytes and platelets in the preparation. The CD45<sup>+</sup> population was further subdivided by the expression of the MHC class II molecule HLA-DR versus lineage markers for B cells (CD19, CD20), T cells (CD3, CD7), and NK cells (CD56). Only a small proportion of the cells were HLA-DR-negative and lineage marker-negative, presumably including granulocytes which had not sedimented through the gradient. Almost half of the cells seemed to be T cells or NK cells (HLA-DR<sup>-</sup> lineage<sup>+</sup>), while B cells (HLA-DR<sup>+</sup> lineage<sup>+</sup>) appeared as a smaller population. None of these populations showed considerable binding of Japanin. About one third of the CD45<sup>+</sup> cells were HLA-DR<sup>+</sup> but did not stain with the lineage markers, therefore presumably comprised monocytes and DC. This population showed indication of Japanin binding. Further subdivision by expression of CD14 versus CD16 identified different subsets of monocytes: Classical monocytes (defined as CD14<sup>++</sup> CD16<sup>-</sup>) represented the majority, followed by intermediate monocytes (defined as CD14<sup>++</sup> CD16<sup>+</sup>), and non-classical monocytes (defined as CD14<sup>+</sup> CD16<sup>++</sup>). Only low binding of Japanin was seen in the monocyte populations, as compared to the binding to moDC (Figure 11). CD45<sup>+</sup> HLA-DR<sup>+</sup> cells which did not stain with lineage markers and did not express CD14 or CD16 were presumed to be DC. This population was therefore divided into four subsets to discriminate between CD1c<sup>+</sup> classical DC, CD123<sup>+</sup> plasmacytoid DC,

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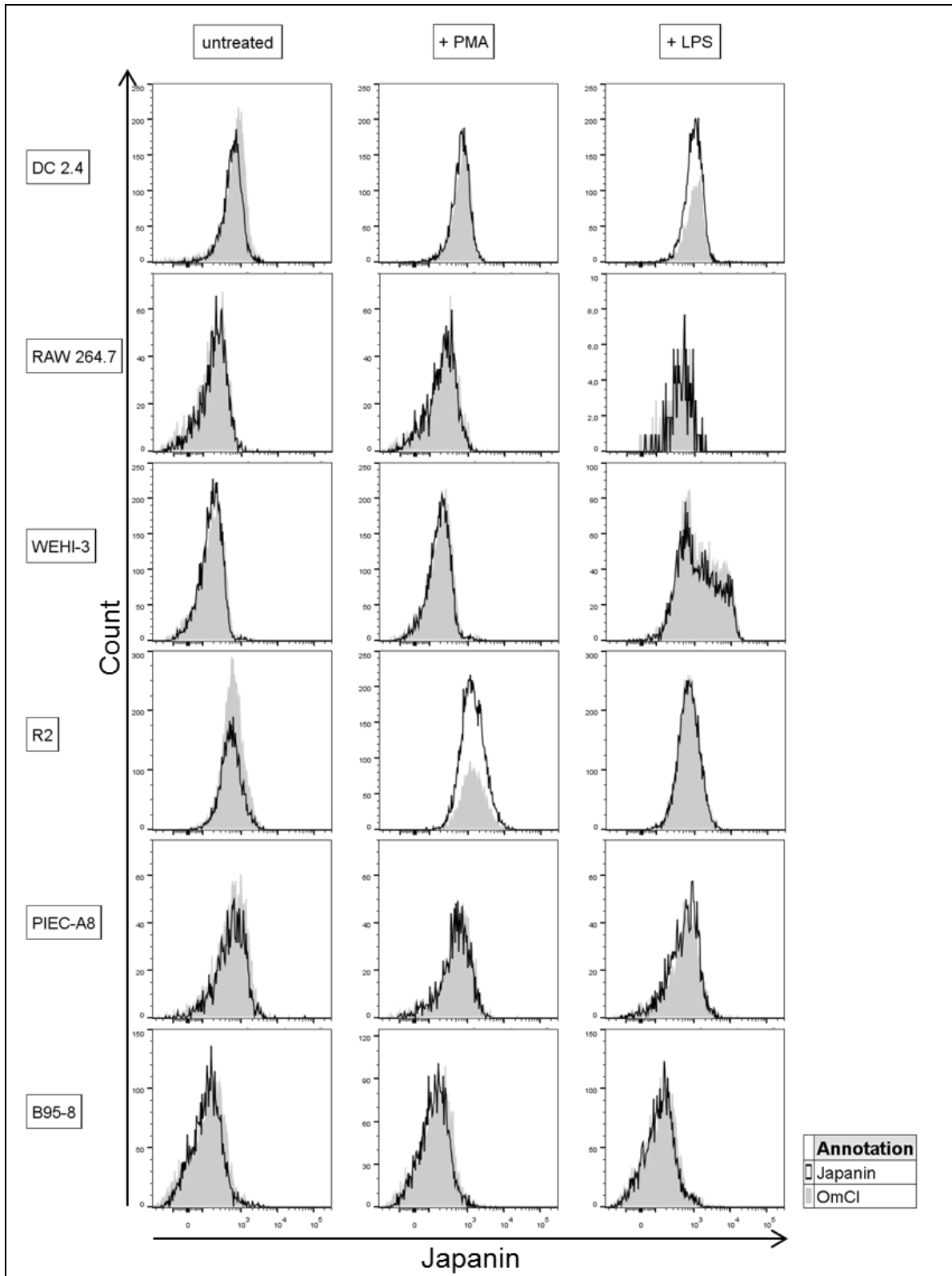
CD11b<sup>+</sup> interstitial DC, and CD141<sup>+</sup> cross-presenting DC. Of all these populations, classical DC showed the most binding of Japanin (Figure 12 B). While the binding to these cells was significant, it was still considerably low compared to moDC (Figure 11). Nevertheless, this staining of different PBMC subsets showed that Japanin most likely bound specifically to DC or DC-precursors, but to no other leucocyte population in human blood. This supported the hypothesis that Japanin bound to DC-restricted receptors, which could also be acquired by monocytes during differentiation into DC.

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*Most DC-like cell lines do not bind Japanin*

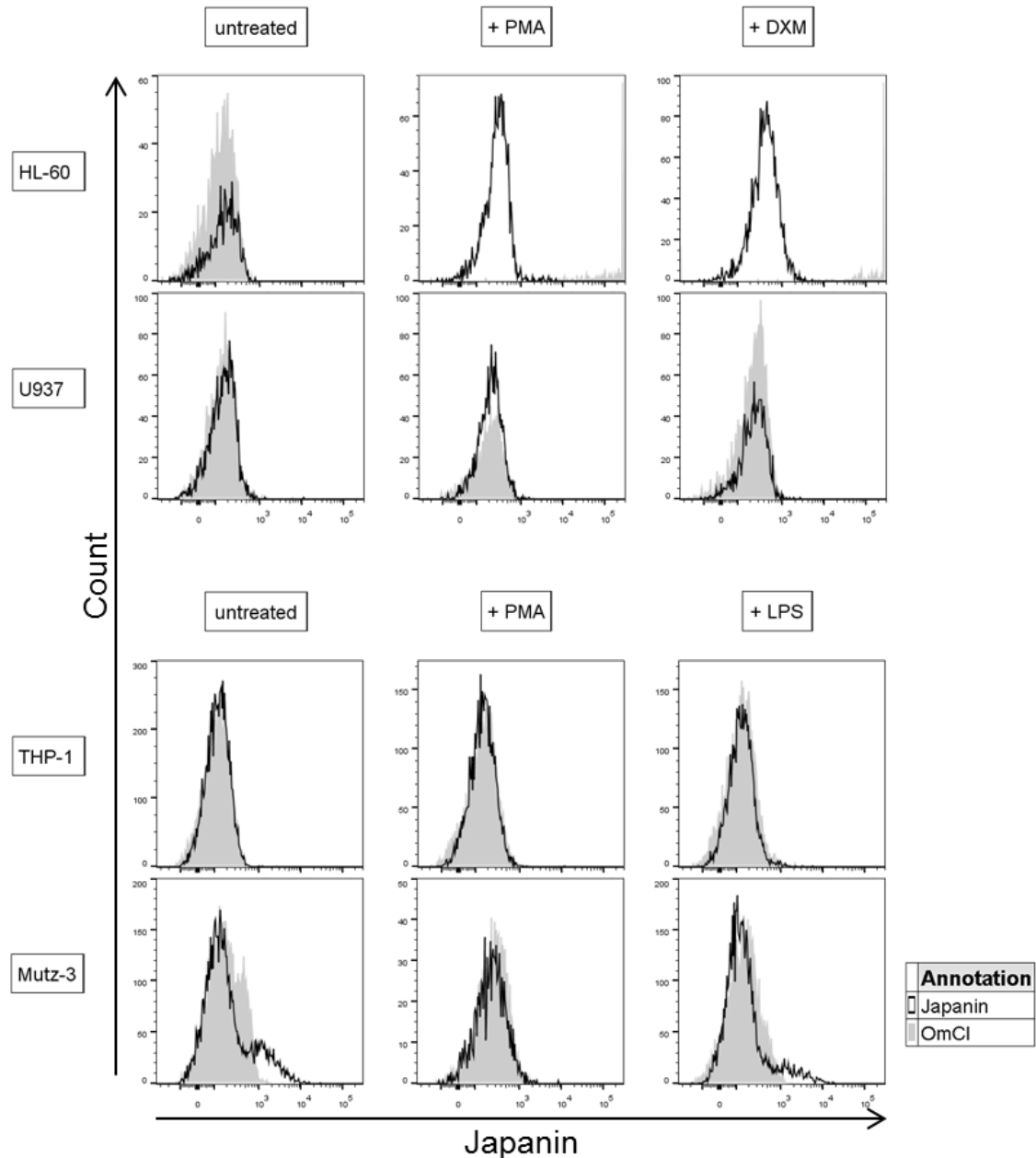
Having found that Japanin specifically binds to monocyte and DC populations, biochemical assays were undertaken to identify the Japanin-binding receptor these cells appeared to express. The establishment of such experiments in our lab required relatively large amounts of cells, but moDC could only be generated in limited numbers since they were derived from donated human blood and cannot be expanded in culture. Hence, we planned to use an established cell line for the experiments, if one could be found that bound Japanin. The binding of Japanin was screened in a number of monocytic or DC-like cell lines from different species, in order to select one which showed detectable binding to an appropriate degree for receptor identification procedures. The acquisition of DC characteristics by monocytic cell lines can be induced by stimulation with PMA, LPS, or dexamethasone (DXM) <sup>302</sup>. Therefore, the binding of Japanin was measured before and after stimulation of the cell lines with these reagents. Whilst it could be shown that Japanin binds highly to moDC and to a lesser extent to monocytes and DC in human blood, surprisingly, no binding of Japanin to any of the tested cell lines was observed, indicating that many mammalian DC-like cell lines do not express the Japanin-binding receptor (Figure 13, Figure 14). The only exception was Mutz-3, a human leukaemia line that spontaneously produces a small proportion of non-proliferating CD14<sup>+</sup>/CD11c<sup>+</sup> DC precursors <sup>302</sup>. This subset may be the population of Mutz-3 cells that showed binding of Japanin (Figure 14).



**Figure 13: Binding of Japanin to different mammalian cell lines.**

Cell lines were cultured overnight, either untreated or stimulated with PMA or LPS at non-toxic dilutions. Fluorescently labelled Japanin or OmCI was applied at 1 µg/ml for 1 hour on ice; cells were washed and the

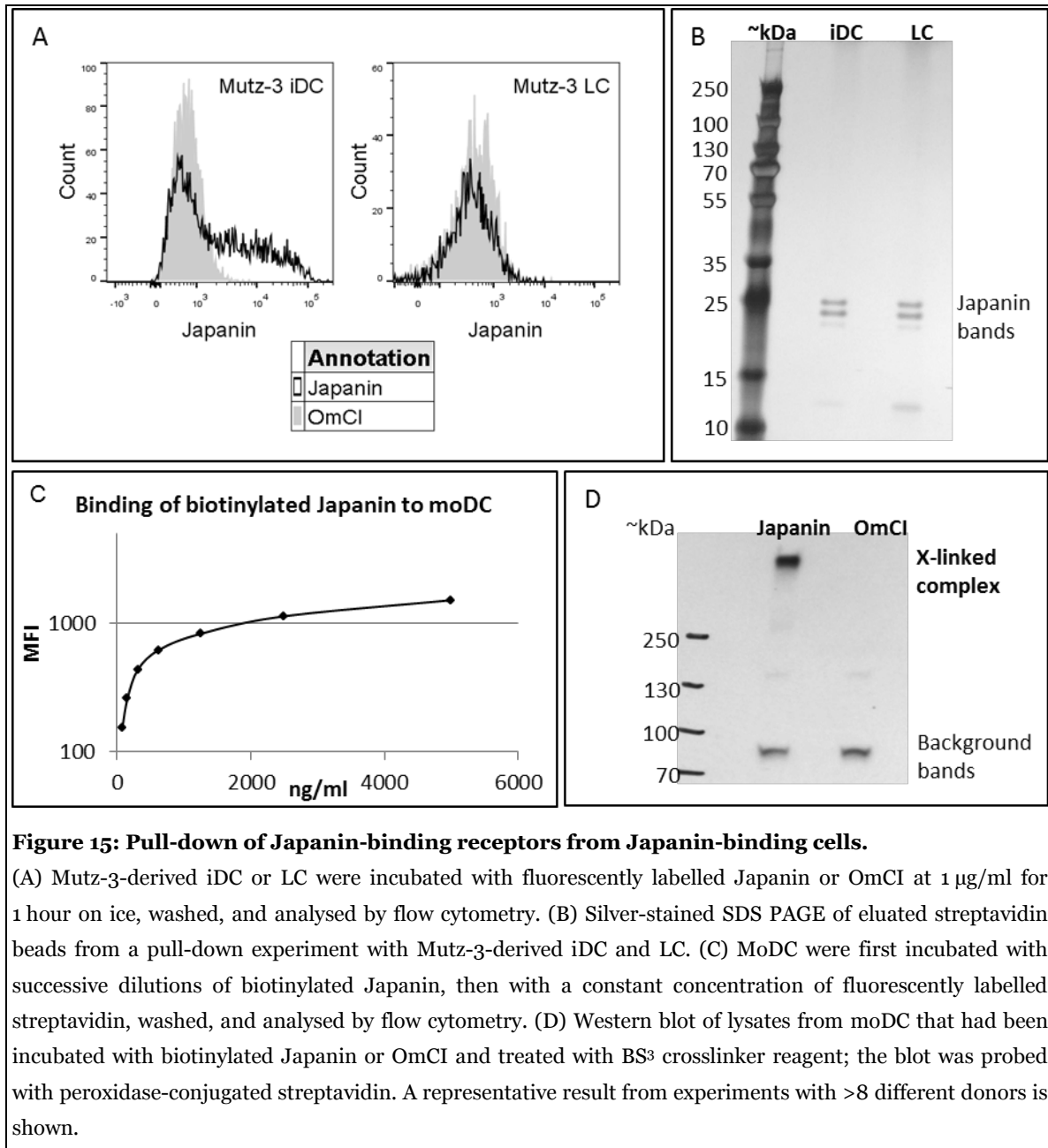
staining analysed by flow cytometry. Each staining was performed in triplicates; representative histograms are shown.



**Figure 14: Binding of Japanin to human cell lines.**

Cell lines were cultured overnight, either untreated or stimulated with PMA, DXM or LPS at non-toxic dilutions. Fluorescently labelled Japanin or OmCI was applied at 1 µg/ml for 1 hour on ice; cells were washed, and the staining analysed by flow cytometry. Each staining was performed in triplicates; representative histograms are shown.

As suggested by Santegoets and colleagues, Mutz-3 might represent the closest DC model within all commonly used cell lines, since they can be differentiated like monocytes into different DC subsets<sup>302</sup>. This distinguishes Mutz-3 from other human leukaemia cell lines. Because the maintenance of Mutz-3 depends on GM-CSF, the culture medium was supplemented with supernatant of a GM-CSF-producing cell line. Further supplementation with TNF- $\alpha$  and IL-4 is reported to induce an interstitial DC (iDC) phenotype, whereas use of TNF- $\alpha$  and TGF- $\beta$  is reported to promote Langerhans cell (LC) differentiation. Only a small proportion of Mutz-3 cultured in GM-CSF-enriched maintenance medium alone bound Japanin (Figure 14). However, when they were cultured under conditions promoting iDC differentiation, the binding increased strongly (Figure 15 A). This suggested that Mutz-3 cells upregulated the expression of Japanin-binding receptors upon differentiation into iDC, similar to monocytes, which acquired the capacity to bind Japanin during the differentiation into moDC. Conversely, differentiation of Mutz-3 cells towards an LC phenotype resulted in diminished binding of Japanin, suggesting that LC do not express Japanin-binding receptors and supporting the hypothesis that Japanin binds specifically to DC.



### 3.2.2 Co-precipitation attempt with Mutz-3-derived iDC and LC

A bead-based pull-down assay, similar to a co-precipitation, was designed with the aim of identifying Japanin-binding receptors on Mutz-3-derived iDC, which had been shown to bind Japanin. This technique involved coupling of Japanin to magnetic beads which were subsequently incubated with cell lysate that was expected to contain the Japanin-specific receptors. This should theoretically enable solubilised receptors to bind to Japanin on the

beads. The bead-bound ligand-receptor complexes could potentially be isolated from the suspension using a magnet, and the receptors might then be identified by subsequent analysis.

In the initial pull-down assay, lysates from Mutz-3-derived iDC were compared to lysates from Mutz-3-derived LC as a negative control. Japanin-coated magnetic beads were incubated in each lysate and then separated from the suspension using a magnet. After washing off unbound proteins from the beads, bound proteins were eluted and analysed by SDS-PAGE and subsequent silver staining. In theory, Japanin-typical protein bands could have appeared in silver-stained SDS gels of both eluates, plus additional bands in the iDC sample only, which could have represented components of Japanin-binding receptors. Indeed, Japanin was detected in both samples, suggesting that the coating of the beads with Japanin had been successful (Figure 15 B). However, there was no difference in the protein band pattern of iDC and LC samples, indicating that no detectable Japanin-specific receptors had been isolated. Formation of Japanin-receptor complexes might have been prevented by detergents in the buffer that was used to lyse the cells. Alternatively, Japanin-receptor ligation might require an intact cell surface, for example in order to allow receptor oligomerisation. To overcome these hindrances, an advanced protocol was used for the following receptor isolation experiments.

### **3.2.3 Identification of potential Japanin-binding receptors**

The next pull-down approach involved cross-linking of Japanin to the cell surface before lysing the cells, with the intention of enhancing the formation of Japanin-receptor complexes by stabilising them during the pull-down procedure. This method, in contrast to the previous assay (3.2.2), did not allow direct coupling of Japanin to magnetic beads.

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Therefore, Japanin was biotinylated, which enabled the use of streptavidin-coated beads that would bind any biotinylated component. The binding of biotinylated Japanin to moDC was confirmed by flow cytometry (Figure 15 C). We decided not to use Mutz-3-derived iDC in this approach, as they turned out to grow only slowly and needed cytokine treatment in order to be useful for pull-down techniques, eventually providing no great advantage over moDC in terms of accessible cell numbers. Hence, we felt it was more appropriate to use moDC. Biotinylated Japanin or OmCI as a negative control were incubated with intact moDC in physiological buffer, washed, and subsequently treated with a cross-linking reagent in order to fixate Japanin to receptors on the cell surface. After cross-linking, the cells were lysed and total cell lysates were analysed by SDS-PAGE and subsequent western blotting. Western blots probed with peroxidase-conjugated streptavidin revealed a component of relatively high molecular weight in the Japanin-treated condition which was absent in the OmCI-treated control, potentially indicating the presence of cross-linked Japanin-receptor complexes (Figure 15 D).

After this result, a pull-down with streptavidin-coated beads was performed in order to separate cross-linked complexes of biotinylated Japanin and corresponding receptors from other proteins in the cell lysates. The beads were first incubated with the cell lysates and then pulled out of the suspension by using a magnet. Subsequently the beads were analysed by mass spectrometry and spectral index normalised quantification, which was performed by Benjamin Thomas, Dunn School of Pathology, University of Oxford. Analyses of two independent experiments demonstrated primarily CD206 / MRC1 in the pull-down (Table 2, Table 3). This was therefore a putative Japanin-binding receptor, and it was exclusively present in the Japanin but not in the OmCI samples. In addition, stabilin-1 (STAB1) / CLEVER-1 / FEEL-1 and CD18 / integrin  $\beta$ 2 (ITB2) were present at

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higher concentrations in the Japanin samples when compared to the OmCI control, though at less abundance than CD206. Based on these results, it was hypothesised that Japanin binds to DC in a CD206-mediated manner, which might also involve binding to stabilin-1 or CD18.

**Table 2: First mass spectrometry results of Japanin receptor pull-down samples**

Protein	Spectral Count		TIC SIn*	
	Japanin	OmCI	Japanin	OmCI
sp P22897 MRC1_HUMAN	34	-	$5,325 \times 10^{-5}$	-
sp Q9NY15 STAB1_HUMAN	1	-	$9,235 \times 10^{-7}$	-
sp P05107 ITB2_HUMAN	1	-	$1,549 \times 10^{-7}$	-

\* Total ion chromatogram normalised spectral index

**Table 3: Second mass spectrometry results of Japanin receptor pull-down samples**

Protein	Spectral Count		TIC SIn*	
	Japanin	OmCI	Japanin	OmCI
sp P22897 MRC1_HUMAN	46	-	$2,309 \times 10^{-5}$	-
sp Q9NY15 STAB1_HUMAN	20	1	$1,949 \times 10^{-6}$	$9,376 \times 10^{-9}$
sp P05107 ITB2_HUMAN	9,96	1	$2,64 \times 10^{-6}$	$3,129 \times 10^{-7}$

\* Total ion chromatogram normalised spectral index

We had been able to detect a strong band in streptavidin-probed western blots of cell lysates from Japanin-treated cross-linking samples, which supposedly contained Japanin-receptor complexes (Figure 15 D). Therefore we attempted to support the mass spectrometry results with methods available in our lab. Proteins were eluted from the streptavidin-beads after pull-down and analysed by SDS-PAGE with subsequent silver staining or western blotting. However, neither Japanin nor any of the proposed receptor candidates could be detected. In order to test whether Japanin was undetectable due to insufficient sensitivity of the anti-his tag antibodies, we compared the sensitivity of anti-

his tag antibodies and streptavidin in western blots with different amounts of biotinylated Japanin. This control experiment indicated that anti-his tag antibodies were less sensitive for Japanin than streptavidin. Therefore, the Japanin protein quantities in the pull-down samples may have been too low for detection by anti-his tag antibodies while sufficient for the detection by streptavidin. As for the lack of detection of the proposed receptor candidates, it is conceivable that the cross-linking masked antibody epitopes crucial for detection or that the elution procedure, which involved denaturing buffer and heat, destroyed such epitopes.

#### 3.2.4 Binding of Japanin correlates with CD206 and stabilin-1 expression

Since mass spectrometry had suggested CD206, stabilin-1 and CD18 as potential receptors for Japanin on DC, investigations were undertaken into whether Japanin actually bound to one, two, or all of the proposed receptors. In order to support or exclude a candidate, receptor expression profiles of moDC and Mutz-3 were generated and compared to those of human monocytic cell lines that did not bind Japanin (Table 4). While CD18 is a cell surface integrin, CD206 and stabilin-1 are recycling receptors mainly located inside the cell. Therefore we measured the expression of CD18 by surface staining following analysis by flow cytometry, whilst detecting CD206 and stabilin-1 in western blots of cell lysates. The binding of Japanin was found to correlate with the expression of CD206 and stabilin-1, whereas CD18 was also expressed by cells which did not show binding of Japanin. Moreover, the failure of Mutz-3-derived LC to bind Japanin was consistent with studies stating that LC are not known to express CD206 on the cell surface, although it has been reported to be detectable at mRNA levels, while CD206 expression is characteristic for moDC<sup>50,302</sup>. This correlation supports the hypothesis of CD206 and/or stabilin-1 as

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Japanin-binding partners, whilst excluding the involvement of CD18 alone. Nevertheless, it was still possible that CD18 might participate as a co-receptor in a CD206- or stabilin-1-mediated binding manner.

**Table 4: Receptor expression profiles of moDC and human DC-like cell lines**

Cell	Japanin binding <sup>#</sup>		CD206 <sup>*</sup>		CD18 <sup>#</sup>		STAB1 <sup>*</sup>	
		PMA		PMA		PMA		PMA
moDC	+	n/a	++	n/a	+	n/a	++	n/a
Mutz-3	+/-	-	+	+/-	+	n/a	+	-
THP-1	-	-	-	-	+ <sup>303</sup>	+ <sup>304</sup>	-	-
HL-60	-	-	-	-	+ <sup>305</sup>	+ <sup>305</sup>	-	-
U-937	-	-	-	-	+ <sup>303</sup>	+ <sup>304</sup>	-	-

<sup>#</sup>data from flow cytometry

<sup>\*</sup>data from western blots

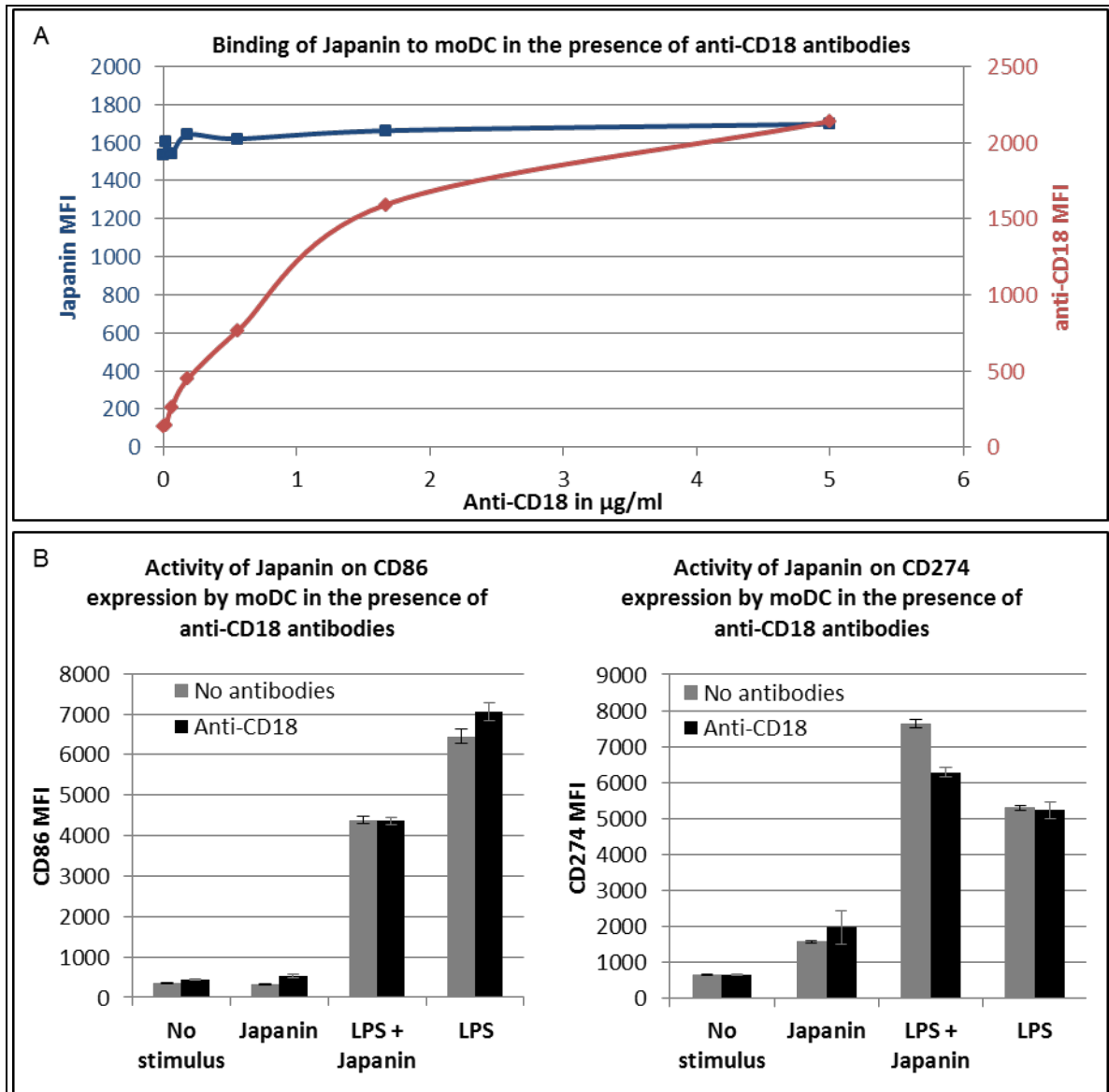
### 3.2.5 Binding and activity of Japanin are not affected by anti-CD18 antibodies

CD18 was suggested as a potential Japanin-binding receptor by the mass spectrometry results upon cross-linking and pull-down of Japanin-receptor complexes, although it was less abundant than CD206 and stabilin-1 (Table 2, Table 3). Moreover, the lack of Japanin binding to cells that expressed CD18 but not CD206 or stabilin-1 showed that CD18 alone was not sufficient to mediate the binding of Japanin. However, CD18 could not be fully excluded from participation in a binding mechanism of Japanin to DC that involved CD206 or stabilin-1. In order to investigate a possible role of CD18 in the binding of Japanin, attempts were made to block interactions of Japanin with moDC by antibodies specific for CD18. As before, flow cytometry binding analysis was performed with fluorescently labelled Japanin or OmCI as a negative control. MoDC were incubated at a

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constant concentration of Japanin in the presence of graded doses of anti-CD18 antibodies. The antibodies used were selected based on reports showing that they could inhibit CD18-mediated chemotaxis<sup>306,307</sup>, suggesting that the same antibodies might generally hinder any ligand interaction. The binding of anti-CD18 antibodies to moDC was verified with secondary antibodies. Ideally, we would have included a labelled known ligand, such as ICAM-1, as a positive control in our assays to confirm whether the anti-CD18 antibodies could actually block the binding of CD18 ligands. If the binding of Japanin was dependent on CD18, increasing anti-CD18 antibody concentration should decrease the Japanin signal. However, the binding of Japanin to moDC was not affected by anti-CD18 antibodies, even at the highest tested antibody concentration (Figure 16 A). Moreover, anti-CD18 antibodies had no effect on the activity of Japanin on moDC, as measured by reduced expression of CD86 and increased expression of CD274 (Figure 16 B). Considering that cells which did not bind Japanin expressed CD18, and that anti-CD18 antibodies did not hinder the binding or activity of Japanin on moDC, an essential role of CD18 in the mode of action of Japanin seemed highly unlikely. Nevertheless, the spatial proximity to the Japanin-receptor complex, as suggested by mass spectrometry, could still indicate involvement in accessory signalling.



**Figure 16: Binding and activity of Japanin are not affected by anti-CD18 antibodies.**

(A) MoDC were incubated with successive dilutions of an anti-CD18 antibodies in the presence of fluorescently labelled Japanin at 500 ng/ml for 1 hour on ice, washed, stained with a fluorescently labelled secondary antibody, and analysed by flow cytometry. The experiment has been performed with cells from 2 different donors; a representative result is shown. (B) MoDC were cultured for 18 hours in the presence or absence of Japanin and/or LPS, with and without addition of anti-CD18 antibodies. The expression of CD86 and CD274 was measured by flow cytometry. Error bars indicate the standard error between triplicates.

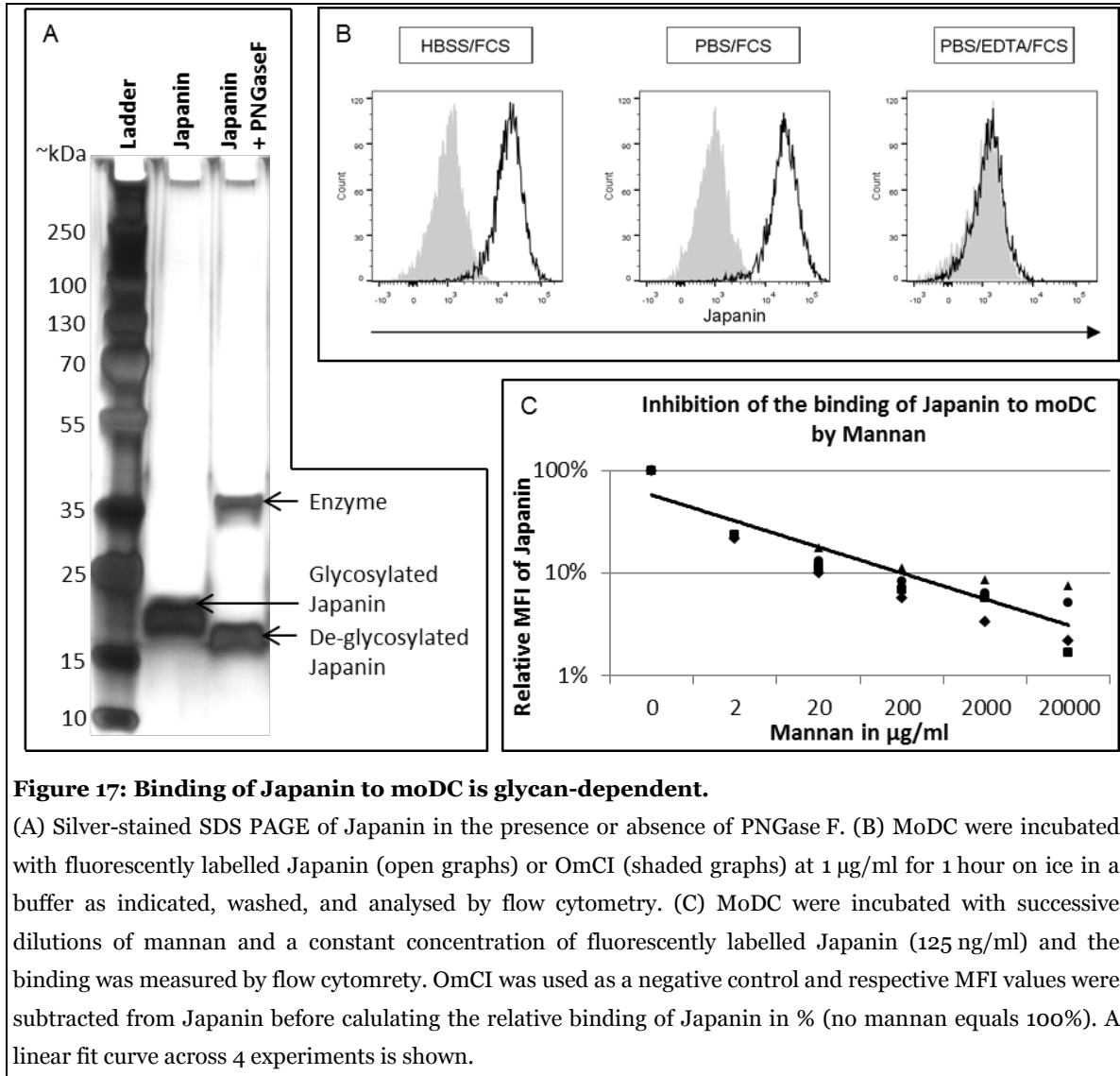
### 3.2.6 Binding of Japanin to moDC is glycan-dependent

Mass spectrometric analysis of pull-down samples upon cross-linking of Japanin to moDC suggested CD206, stabilin-1 and CD18 as potential binding partners of Japanin. Having

excluded an essential role of CD18 in the binding of Japanin to DC, attempts were made to confirm whether Japanin bound one or both of the remaining receptor candidates, CD206 and stabilin-1.

#### *Binding of Japanin to moDC can be inhibited by mannan and EDTA*

CD206 contains multiple Ca<sup>2+</sup>-dependent CTLDs (Figure 1) and therefore binds the polysaccharide mannan with high affinity<sup>180</sup>. Stabilin-1 on the other hand, contains only a rudimentary lectin-like link which is considered non-functional<sup>308</sup>. In order to assess the respective roles of CD206 and stabilin-1 in the binding of Japanin to DC by their differential lectin-like activity, Japanin binding competition and inhibition studies were undertaken. Competitive binding experiments were performed with mannan, as this polysaccharide binds strongly to CD206. In brief, the binding assays were carried out as before, but with a constant concentration of fluorescently labelled Japanin or OmCI and in the presence of successive dilutions of mannan. Flow cytometric analysis showed that the binding of Japanin to moDC decreased with increasing concentrations of mannan, with the highest mannan concentration tested reducing binding by ~98% (Figure 17 C). We also investigated the binding of Japanin to moDC in the presence of EDTA in physiological buffer. This chelating agent was used as an inhibitor because of its ability to capture metal ions, such as Ca<sup>2+</sup>, which are crucial for carbohydrate binding by C-type lectins. EDTA abolished the binding of Japanin to moDC almost completely, suggesting a Ca<sup>2+</sup>-dependency (Figure 17 B). The drastic decrease in the binding of Japanin to moDC upon mannan and EDTA treatment collectively indicated that the binding was carbohydrate-dependent and therefore strongly suggested that CD206, but not stabilin-1, was the major Japanin-binding receptor on DC.



### *Glycosylation of Japanin is crucial for stability*

Mannan competition and EDTA inhibition assays suggested that Japanin might bind to the active CTLD of CD206, which is CTLD4<sup>309</sup>. Therefore, we next attempted to investigate whether this binding was mediated by glycans on Japanin. We previously demonstrated that Japanin could be expressed with different extents of glycosylation, depending on the cell used for expression<sup>310</sup>. Japanin produced by mammalian cells was found to have a higher molecular weight than the same protein produced by insect cells, but both forms were active, as shown by decreased expression of CD86 in the presence of

LPS. Since even with less glycosylation Japanin was able to decrease CD86 expression by moDC, glycans might not be essential for the activity and consequently the binding of Japanin. Alternatively, we have previously proposed that Japanin may bind to distinct receptors in carbohydrate-dependent and carbohydrate-independent fashions, respectively accounting for different functions of Japanin <sup>310</sup>.

In order to investigate whether the glycosylation of Japanin was crucial for its binding to CD206, competitive binding and activity tests were planned with glycosylated Japanin in comparison to mutated versions of the protein in which the glycosylation motifs were destroyed. In our previous work, we had mutated one of the two glycosylation motifs in the Japanin sequence at a time and could demonstrate that either one of the two glycosylation sites was sufficient for the activity of Japanin <sup>310</sup>. However, these studies could not show whether glycosylation was actually required in this context. Competitive assays with glycosylated and non-glycosylated Japanin might reveal whether activity and binding relied on carbohydrates. Exclusive binding of glycosylated Japanin would indicate a glycan-dependency, whereas binding of non-glycosylated Japanin would suggest a different binding pattern. Non-glycosylated Japanin would most likely bind to a different domain than mannan, which binds to CTLD4. However, large sugar polymers might still sterically prevent the binding of Japanin to another domain. Alternatively, non-glycosylated Japanin might bind to the same domain as mannan but in a carbohydrate-independent fashion, which would represent a novel binding pattern for CD206. Baculovirus encoding Japanin with mutations in both glycosylation motifs was amplified in order to produce non-glycosylated Japanin protein in insect cells. However, transfected insect cell cultures did not express this mutated version of Japanin. Our previous studies had shown that Japanin with mutations in both glycosylation sites could be expressed by

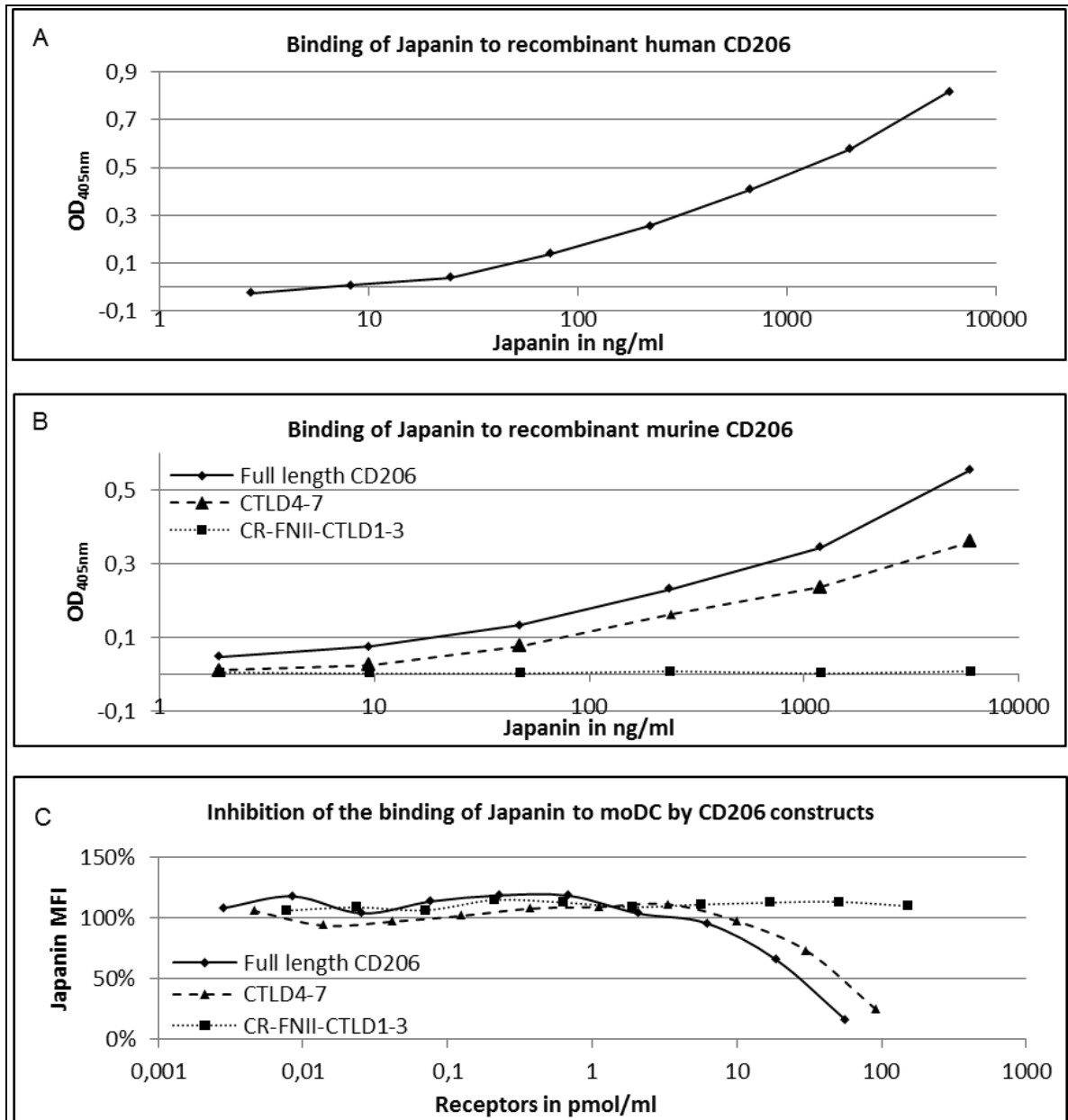
transfected mammalian cells <sup>310</sup>. Yet these transfections did not produce Japanin in sufficient quantities for binding or activity assays with purified protein. Besides, the expression of non-glycosylated Japanin was not reproducible within this study. The introduction of two mutations at a time may impair protein biosynthesis of Japanin <sup>311</sup>. This would be consistent with observations from previous studies, that the amounts of expressed Japanin decreased when one glycosylation motif was mutated at a time and it decreased even more when two mutations were introduced <sup>310</sup>. The extent of the decrease depended moreover on the mutated residue and the cell line used for protein expression, suggesting that it was the mutation itself, rather than the absent glycosylation, that hindered expression.

As an alternative approach, attempts were made to produce a non-glycosylated version of Japanin by enzymatic deglycosylation. Japanin was therefore treated with PNGase F, an enzyme that cleaves most N-linked glycans from proteins, except for some plant and insect glycoproteins with an  $\alpha$ 1-3-linked fucose core <sup>312</sup>. Following PNGase F-treatment, Japanin was analysed by SDS-PAGE and protein bands were visualised by silver staining. A reduction in the molecular weight of Japanin indicated that its glycans had been cleaved (Figure 17 A). In order to purify deglycosylated Japanin from the cleaved glycans and any residual glycosylated protein, attempts were made to bind unwanted glycan components onto a concanavalin A (ConA) column. Deglycosylated protein was in fact collected in the flow through fraction, but it was unstable and precipitated in physiological buffer, and could therefore not be used in further studies. The instability of deglycosylated Japanin suggested that glycosylation was crucial for the stability of the protein. Hence no binding or activity experiments could be undertaken with non-glycosylated Japanin. The instability of deglycosylated Japanin might also explain why no Japanin could be obtained

from bacterial cultures in the initial expression attempts by others, since bacterial systems that are able to produce N-glycosylated proteins have only recently been identified and were not available to our lab then <sup>313</sup>. However even if this method had been available, we might have preferred the insect cell expression system still, because it minimises the risk of endotoxin contaminations in Japanin preparations. We aim to avoid endotoxins as they would potentially interfere with the modulatory functions of Japanin in moDC activity assays and could moreover hinder future in vivo testing as they may cause sepsis.

### 3.2.7 Japanin binds to recombinant human and mouse CD206

The apparent glycan-dependency in the binding of Japanin to moDC suggested that CD206 was the major Japanin-binding partner on DC. In order to confirm that Japanin bound to CD206, plate-based binding assays were carried out with the recombinant receptor instead of DC. These experiments involved immobilisation of a constant amount of recombinant CD206 on a microtitre plate that was subsequently incubated with graded doses of Japanin, to assess dose-dependent binding. Biotinylated Japanin was used since this approach enabled photometric detection. The binding was visualised by addition of peroxidase-conjugated streptavidin plus a peroxidase substrate that developed an optically measurable end product. Japanin was found to bind to both recombinant human and mouse CD206 in dose-dependent fashions (Figure 18 A+B). This showed that CD206 was able to bind Japanin without involvement of other receptors.

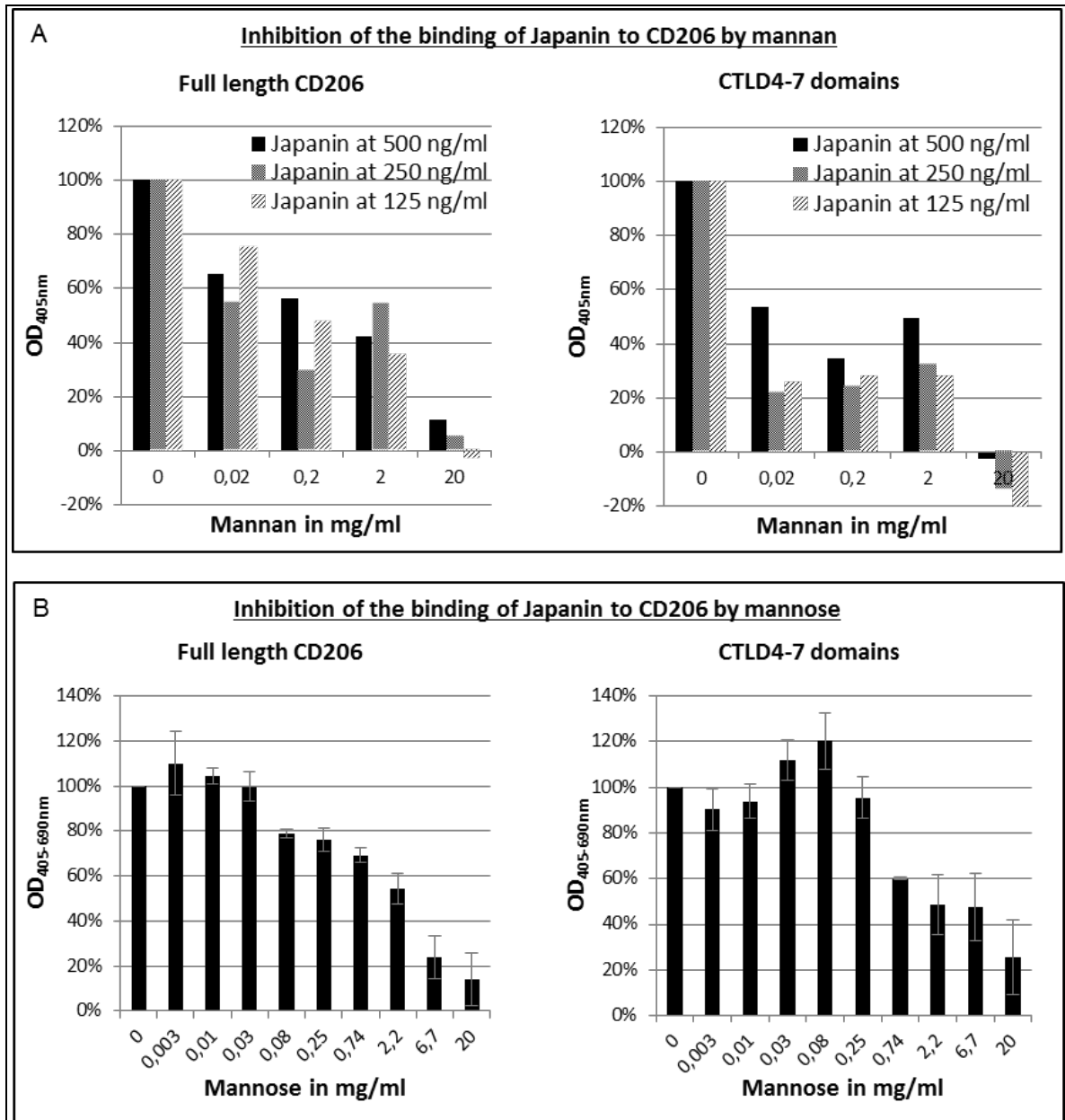


**Figure 18: Japanin binds to recombinant CD206.**

(A+B) Recombinant (A) human or (B) mouse CD206 constructs were immobilised on a microtitre plate and incubated with grades doses of biotinylated Japanin. Binding was measured by photometry after incubation with streptavidin-peroxidase and a suitable substrate for detection. OmCI was used as a negative control and respective OD values were subtracted from Japanin. Representative results from >3 independent experiments are shown. (C) Graded doses of soluble full length mouse CD206 or truncated constructs were added to an moDC suspension with fluorescently labelled Japanin. Receptors were successively diluted starting from 56 pmol/ml of full length CD206, 91 pmol/ml of CTLD4-7, and 154 pmol/ml of CR-FN-II-CTLD1-3, while Japanin was constantly at 28 pmol/ml (0.5 µg/ml). Subsequently, moDC were incubated with the constructs and Japanin at 4 °C for 1 h. The binding of Japanin to moDC was measured by flow cytometry. 100% marks the binding of Japanin in the absence of any receptors. All values include subtraction of the OmCI control. Only one experiment was performed, due to limited obtainability of recombinant CD206 constructs.

*Japanin binds to the carbohydrate-binding domain of CD206*

Since the instability of deglycosylated Japanin did not allow binding competition assays with glycosylated and non-glycosylated Japanin, a different approach was needed to investigate the carbohydrate-dependency of the binding of Japanin to CD206. In addition to the full length mouse CD206 molecule, Luisa Martinez-Pomares at the University of Nottingham kindly provided us also with truncated versions of the receptor, containing either the CR-FNII-CTLD1-3 or the CTLD4-7 domains. These constructs enabled a comparison of the binding of Japanin to CD206 domains that have been reported to bind different kinds of ligands. Using the plate assay described above, it was shown that Japanin bound to the CTLD4-7 but not to the CR-FNII-CTLD1-3 domains (Figure 18 B). This was consistent with the observation that the binding of Japanin could be inhibited by mannan and EDTA, since CTLD4 is thought to be the active carbohydrate-binding domain of CD206 <sup>309</sup>. In analogy to the competitive binding experiment with DC, competition of the binding of Japanin to recombinant CD206 or the truncated domains by mannan was assessed in plate-based assays with immobilised receptors. The binding of Japanin to both the recombinant full length CD206 and the truncated CTLD4-7 construct was gradually impaired by increasing doses of mannan (Figure 19 A). In order to address the possibility that the binding of Japanin to a domain other than CTLD4 might be sterically prevented by large mannan molecules, monomeric mannose was additionally used as a competitor for Japanin in this experiment. Mannose was also found to be a potent competitor for the binding of Japanin to CD206 and the CTLD4-7 domains of the receptor, suggesting that steric hindrance did not play a role in the inhibition of the binding (Figure 19 B). These findings strongly suggested that Japanin binds to CTLD4, the active carbohydrate-binding domain of CD206.



**Figure 19: Inhibition of the binding of Japanin to CD206 by mannan and mannose.**

Recombinant mouse full-length CD206 or CTL4-7 domains were immobilised on a microtitre plate and incubated with successive dilutions of (A) mannan or (B) mannose. Biotinylated Japanin was added (A) as indicated or (B) at 250 ng/ml. The binding of Japanin was detected by photometry after incubation with streptavidin-peroxidase and a suitable substrate for optical detection. OD values of OmCI controls were subtracted from Japanin. Representative experiments from 3 independent repeats are shown. (B) Error bars indicate standard error between duplicates.

To further confirm that CD206 is a potent binding partner for Japanin, a competition experiment with DC was set up. In this assay, either the soluble full length CD206 molecule or the truncated domain constructs were used as competitors to inhibit the

binding of Japanin to cell-associated receptors. Both CD206 and the CTLD4-7 construct drastically decreased the binding of Japanin to moDC in a dose-dependent fashion, while the CR-FNII-CTL1-3 construct did not inhibit the binding (Figure 18 C). These results lend further support to the hypothesis that the carbohydrate-binding domain of CD206 was the Japanin binding partner on moDC.

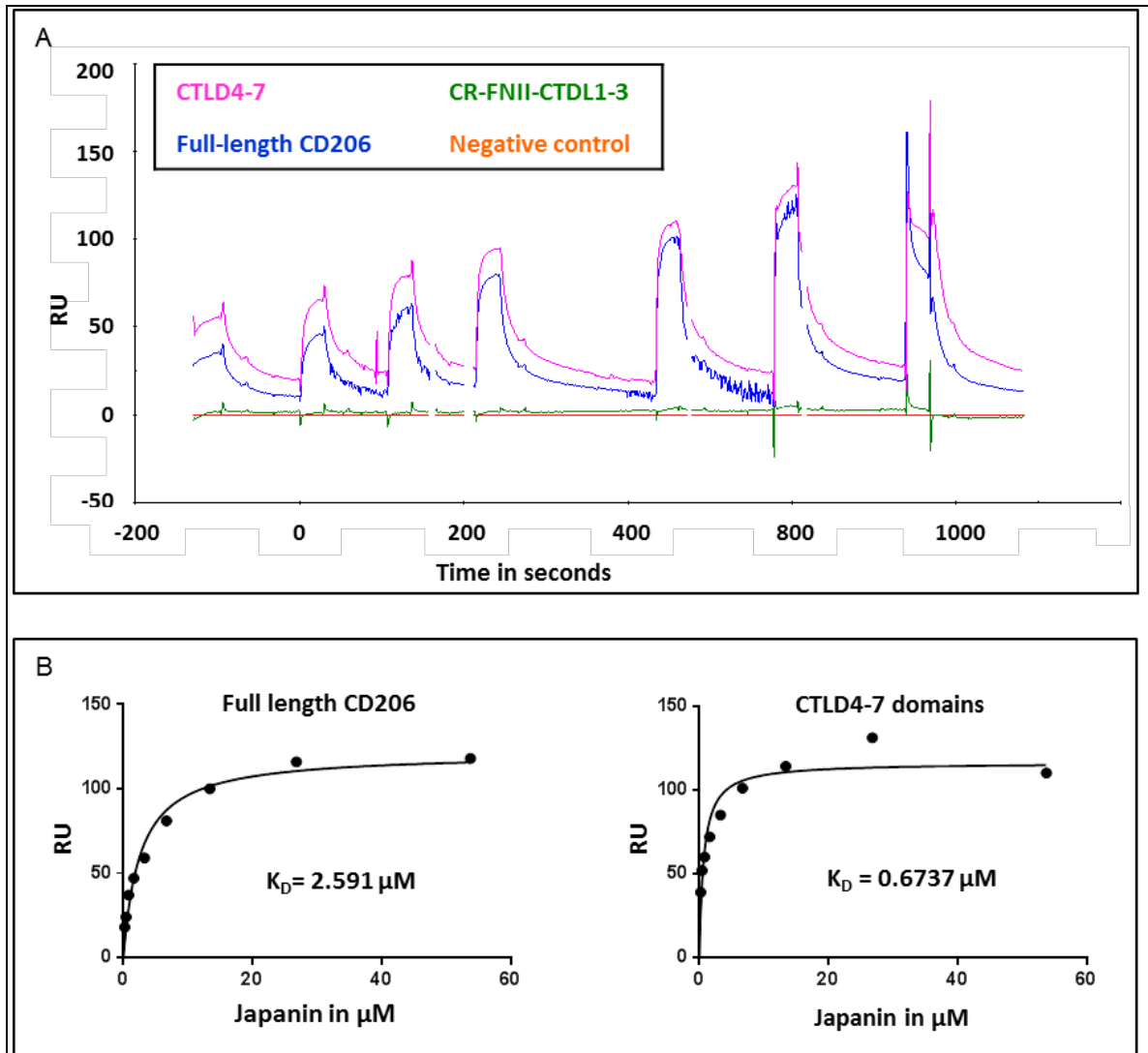
#### *Japanin binds to CD206 with relatively low affinity*

Plate-based binding experiments with photometric read-out showed that CD206 and its CTLD4-7 domains bound biotinylated Japanin. To determine the affinity of the binding and to verify our simple assay with a more sophisticated approach, surface plasmon resonance (SPR)-based analysis was performed using a Biacore instrument. The SPR technique enabled acquisition of real time binding data that could be used to assess the strength of the binding. Another advantage of this optical detection method was that it did not require labelling of Japanin. Murine full-length CD206, CTLD4-7 domains, CR-FNII-CTL1-3 domains, and streptavidin as a negative control, were immobilised onto individual flow cell surfaces of a sensor chip. Successive dilutions of Japanin were then injected into the system and passed through the chip. The binding of Japanin to receptors in the flow cell resulted in an increased refractive index, which was plotted in a sensorgram as response units (RU) over time (Figure 20 A). Japanin strongly bound to full length CD206 and the CTLD4-7 domains, while binding to the CR-FNII-CTL1-3 domains did not significantly increase compared to the negative control. After subtraction of the streptavidin response, binding curves of the binding of Japanin to CD206 and to the CTLD4-7 construct were plotted as RU versus the concentration of Japanin in  $\mu\text{M}$  (Figure 20 B). These plots were used to generate dissociation constants ( $K_D$ ), which were  $2.591 \mu\text{M}$  for full length CD206 and  $0.6737 \mu\text{M}$  for the CTLD4-7 domains, indicating a slightly

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higher affinity for the latter. However, these numbers were calculated based on the assumption of a 1:1 stoichiometric ratio of Japanin and receptors during the binding, which might not to be true since Japanin forms dimers. Other factors weakening the accuracy of these data were that the low end of the titration was not fully mapped and the injection times were very short due to limited sample volume. Although the calculated binding affinity was not perfectly accurate, the Biacore analysis was consistent with our plate binding assays, verifying their results.



**Figure 20: Biacore analysis of the binding of Japanin to CD206.**

Each flow cell of the sensor chip was coated with either recombinant mouse full length CD206, a truncated CD206 construct (CTLD4-7 or CR-FNII-CTLD1-3), or streptavidin as a negative control ligand. Successive dilutions of Japanin were injected for 30-60 seconds at 10  $\mu\text{l}$  per minute, starting with the highest dilution. OmCI was injected as a negative control and did not show specific binding (data not shown). (A) Sensorgram of Japanin binding to ligands on the flow cell, measured in response units (RU) over time. (B) Plots of the binding of Japanin to full length CD206 and the CTLD4-7 domain construct as RU over the molar concentration of Japanin. The dissociation constant ( $K_D$ ) for the binding to full length CD206 is 2.591  $\mu\text{M}$ , for the CTLD4-7 construct the  $K_D$  is 0.6737  $\mu\text{M}$ . The system was operated and its raw data were analysed with kind help from Marcus Bridge at the Dunn School of Pathology, University of Oxford. The experiment was performed once.

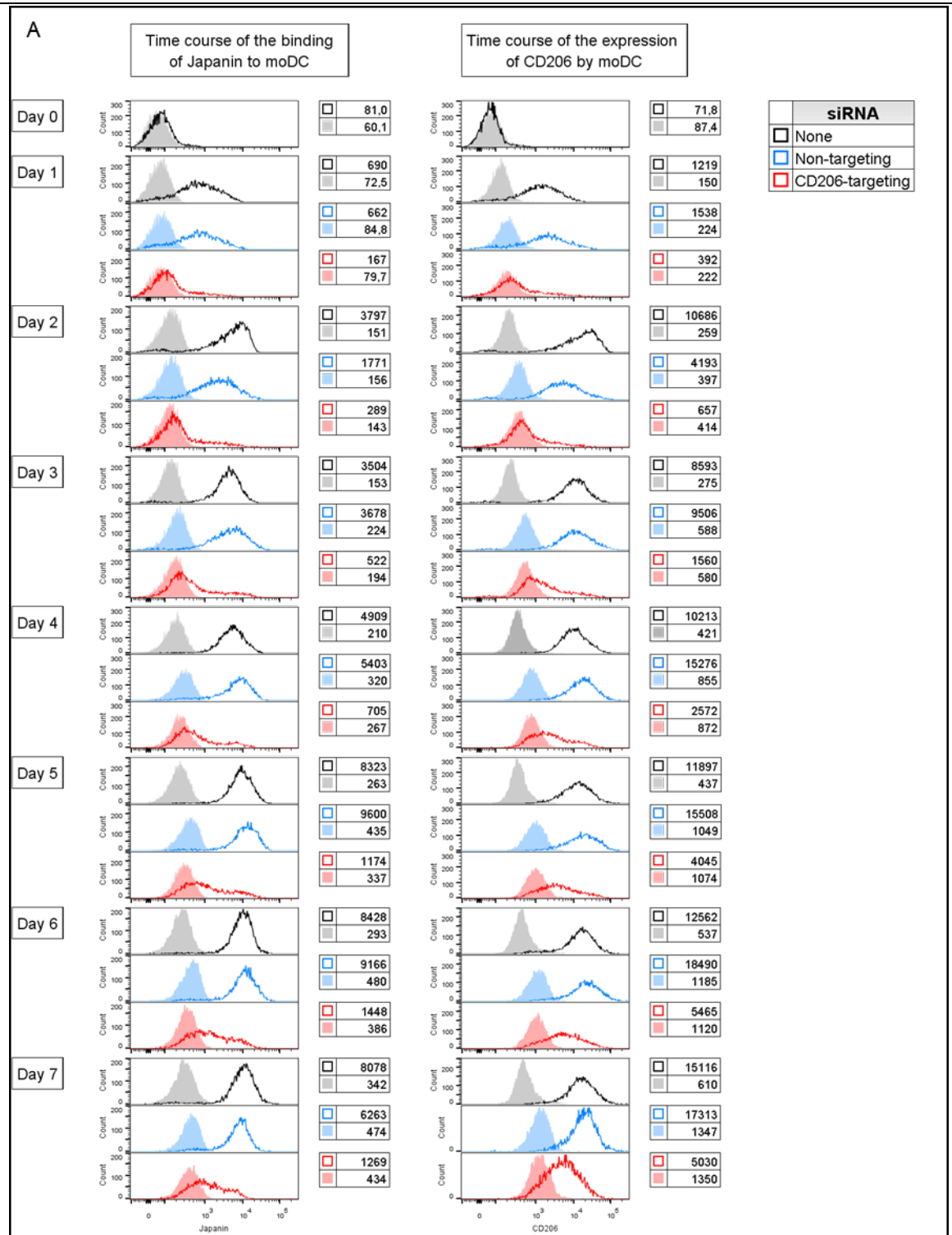
### 3.2.8 CD206 deficiency results in diminished binding of Japanin

Having shown that the binding of Japanin to moDC is glycan dependent and that Japanin binds to recombinant CD206, we intended to confirm that the binding of Japanin to moDC was mediated by this receptor.

#### *DC and macrophages from CD206 KO mice bind less Japanin than WT cells*

Since plate-based binding assays and Biacore analysis indicated that Japanin bound to mouse CD206, which is expressed by BMDC as well as bone marrow-derived macrophages (BMM), both cell types were generated for binding studies. Bone marrow of CD206 knock-out (KO) mice and corresponding wild-type (WT) mice, potentially expressing CD206, was received as a kind gift from Luisa Martinez-Pomares at the University of Nottingham. Recombinant murine GM-CSF or M-CSF was used to differentiate BMDC or BMM, respectively. After 7 days of culture, the expression of CD206 and the binding of Japanin were assessed by flow cytometry (Figure 21 B). As was observed previously, the binding of Japanin correlated with the expression of CD206, as WT BMM expressed the most CD206 and also bound the most Japanin. On the contrary, the expression of CD206 and the binding of Japanin by knock-out BMM were significantly decreased, suggesting that the loss of CD206 disabled the binding of Japanin. WT BMDC expressed only low levels of CD206, comparable to knock-out BMM, and likewise bound less Japanin. Since the expression of CD206 by WT BMDC was already very low, no significant decrease in the expression of CD206 was observed in knock-down BMDC. However, a decrease in the binding of Japanin in CD206 knock-out BMDC when compared to WT BMDC, might suggest that Japanin may be a more sensitive or more specific marker for CD206 than the anti-CD206 antibody used in this experiment.

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**Figure 21: Knock-down of CD206 results in diminished binding of Japanin.**

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**Figure 21: Knock-down of CD206 results in diminished binding of Japanin.**

(A) Human monocytes were isolated from PBMC and immediately transfected by electroporation with CD206-specific siRNA (red). As negative controls, electroporation was also performed with non-targeting siRNA (blue), or monocytes were not electroporated nor siRNA treated (black). Differentiation into moDC was induced by IL-4 and GM-CSF in the culture medium. The binding of Japanin and the expression of CD206 were monitored daily by flow cytometry. MFI values are indicated in the box to the right of the respective histograms. Shaded histograms indicate OmCI or isotype controls. The experiment was performed with moDC from 2 different donors; representative results are shown. (B) Bone marrow cells from CD206 KO and WT mice were cultured in the presence of GM-CSF or M-CSF in order to induce differentiation into BMDC or BMM, respectively. The binding of Japanin and the expression of CD206 were measured after 7 days of culture by flow cytometry. The experiment was conducted twice; representative results are shown; error bars indicate the standard error between triplicates.

*Knock-down of CD206 in human moDC decreases the binding of Japanin*

It was previously observed that the binding of Japanin increased during the differentiation of monocytes into DC<sup>290</sup>. Likewise, the expression of CD206 is upregulated by monocytes as they differentiate into DC. Due to this correlation, we hypothesised that diminished expression of CD206 during the differentiation of moDC would result in decreased binding of Japanin, if it was mediated by CD206. In order to test this hypothesis, monocytes were transfected by electroporation with siRNA targeting CD206. CD206 knock-down protocols developed by others suggested an siRNA treatment of moDC on day 2 of the differentiation culture<sup>314</sup>. However, the experiments in our lab had shown that the binding of Japanin was already detectable from day 1 of the moDC culture, indicating that the expression of Japanin-binding receptors started from the beginning of the culture. Hence we decided to knock-down the expression of CD206 in monocytes directly after isolation, before adding cytokines for differentiation into moDC. As a control for knock-down specificity, non-targeting siRNA was inserted into monocytes by electroporation. Non-electroporated monocytes were a positive control for unrestricted expression of CD206 and binding of Japanin. Monocytes of each condition were cultured for a week in the presence of GM-CSF and IL-4 to induce differentiation into DC. The

expression of CD206 and the binding of Japanin were meanwhile monitored daily by flow cytometry. Non-electroporated moDC and those treated with non-targeting siRNA upregulated CD206 steadily until day 5 whilst at the same time the binding of Japanin increased (Figure 21 A). On the contrary, the majority of moDC transfected with CD206-targeting siRNA showed significantly reduced expression of CD206 over the course of differentiation, indicating that the knock-down of CD206 had been successful. A simultaneous decrease in the binding of Japanin by CD206-deficient moDC strongly suggested that the binding of Japanin to moDC was mediated by CD206.

### 3.2.9 Summary of chapter 3.2

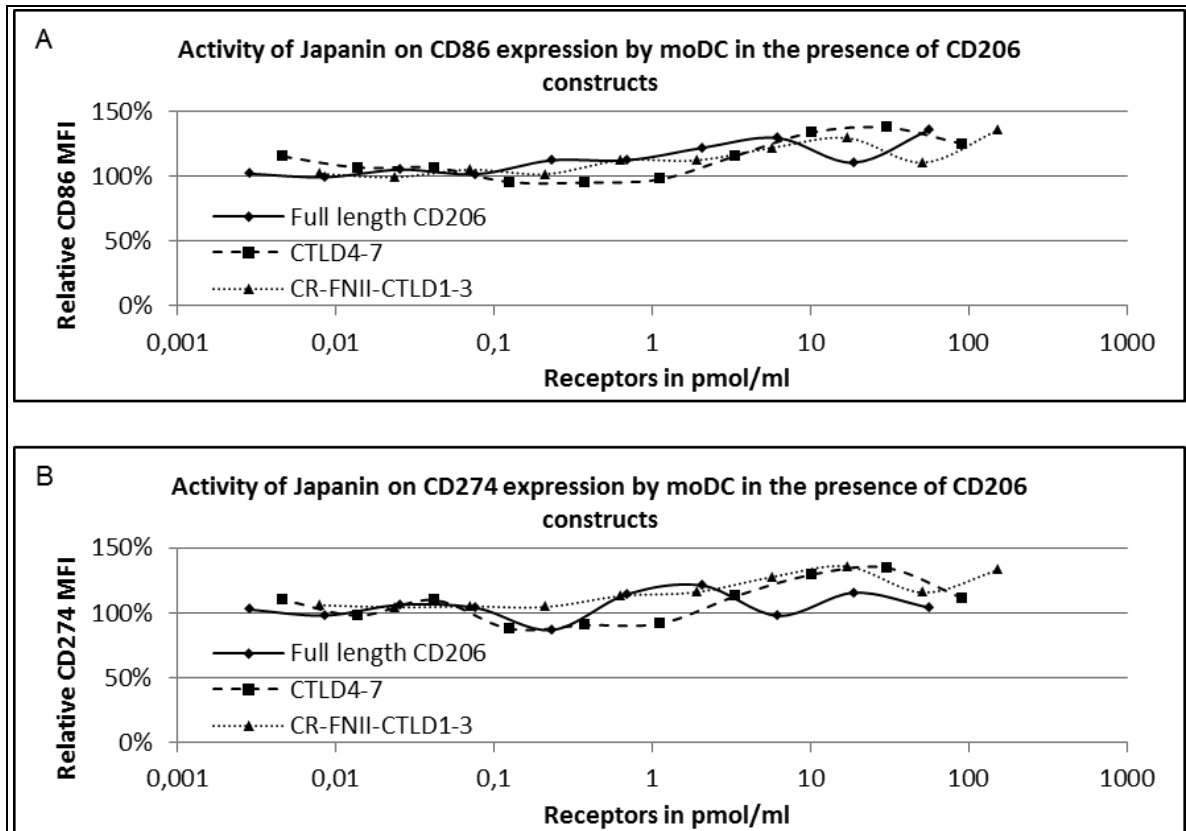
This chapter was set out to identify specific Japanin-binding receptors on DC, which would potentially mediate the immunomodulatory effects of Japanin. Fluorescent labelling of Japanin revealed that it bound specifically to moDC as well as monocytes and DC in human blood, but no other major leucocyte population. Nor was there any detectable binding of Japanin to commonly used monocytic or DC-like cell lines, except Mutz-3. Pull-down experiments suggested CD206, stabilin-1, and CD18 as potential Japanin-binding partners on DC. Subsequent blocking, competition and inhibition assays showed that the binding of Japanin to moDC was not impaired by anti-CD18 antibodies, but by mannan and EDTA. The latter indicated a glycan-dependency of the binding and thereby suggested CD206, not stabilin-1, as a Japanin binding partner. The capacity of CD206 to bind Japanin was confirmed by studies with recombinant receptor constructs, which also revealed that Japanin bound to the CTLD4-7 domains of CD206. Eventually, experiments with CD206-KO BMDC and BMM as well as CD206 knock-down moDC strongly suggested that the binding of Japanin to DC was mediated by CD206.

### 3.3 The role of CD206 in the activity of Japanin

Having identified CD206 as a Japanin-binding receptor on DC in the previous chapter, we aim to investigate its role in the mechanism of action of Japanin in the following chapter. Therefore we will evaluate whether Japanin may modulate DC that lack expression of CD206.

#### 3.3.1 The activity of Japanin is not inhibited by CD206 constructs

Based on the identification of CD206 as a Japanin-binding receptor on DC in chapter 3.2.3, we hypothesised that this receptor would also be involved in the activity of Japanin. Having successfully used recombinant soluble full length CD206 and CTLD4-7 domain constructs in a microtitre assay to prevent the binding of Japanin to moDC (Figure 18), we now aimed to investigate whether these constructs were also able to inhibit the modulatory functions of Japanin. Therefore, moDC were stimulated in microtitre plates with constant concentrations of LPS and Japanin in the presence of graded doses of soluble full length CD206 or its CR-FNII-CTL1-3 or CTL4-7 domain constructs. Relative decrease or increase in the expression of CD86 or CD274 was used respectively as a measure for the activity of Japanin. Interestingly, the receptors that had previously prevented the binding of Japanin to moDC did not inhibit the modulatory functions of Japanin on moDC (Figure 22).



**Figure 22: The activity of Japanin on moDC is not inhibited by recombinant soluble CD206 constructs.**

MoDC were incubated with graded doses of recombinant soluble mouse full-length CD206 or domain constructs in the presence of Japanin and/or LPS. Japanin was at 28 pmol/ml (0.5 µg/ml) while receptors were successively diluted starting from 56 pmol/ml of full length CD206, 91 pmol/ml of CTLD4-7, and 154 pmol/ml of CR-FN-II-CTLD1-3. The assay was initially incubated for 24 hours at 37 °C without LPS, then additional 18 hours with LPS. The expression of (A) CD86 and (B) CD274 was measured by flow cytometry; 100% marks the expression in the absence of any receptors. The experiment was conducted once due to limited obtainability of recombinant CD206 constructs.

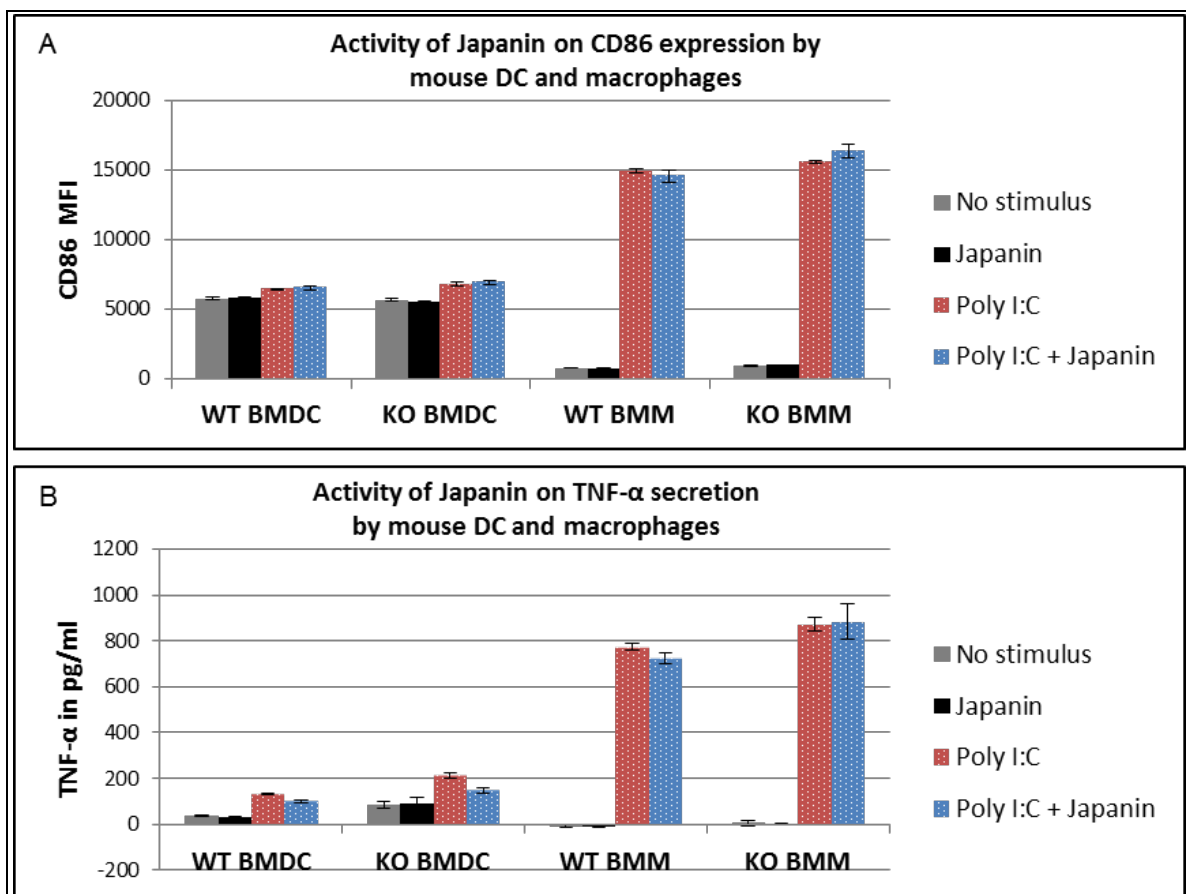
Complexes of Japanin and soluble constructs might have dissociated over time, allowing the binding of liberated Japanin to the cells during the rather long incubation of 2 days at 37°C in this assay. The binding of Japanin to moDC could have been tested with labelled Japanin in the presence or absence of CD206 constructs under these conditions. Another possibility to explain this phenomenon could be that the constructs were not stable in the culture. By contrast, the previous binding experiment had been performed for 1 hour at 4°C. However, it was not possible to measure the activity of Japanin under these

conditions, since the expression of CD86 and CD274 would not be detectable. We therefore intended to undertake further investigations into the potential role of CD206 in the mode of action of Japanin that would provide less ambiguous results.

### 3.3.2 CD206 alone is not sufficient for modulation by Japanin

After attempts to inhibit the activity of Japanin by blocking its binding to moDC with recombinant CD206 constructs, we next took a different approach to determine whether or not the modulatory functions of Japanin were mediated by this receptor. In chapter 3.2.8 we had shown that CD206 deficiency diminished the capacity of cells to bind Japanin. Mouse BMM differentiated from CD206 KO bone marrow had been demonstrated to bind less Japanin than corresponding WT BMM, which expressed high levels of CD206 (Figure 21 B). BMDC on the other hand naturally expressed only low levels of CD206, consequently there was only very little binding of Japanin to these cells. Preliminary experiments with mouse BMM conducted by others in our lab had indicated that Japanin decreased the expression of CD86 and TNF- $\alpha$  by BMM; yet in contrast to moDC only upon stimulation with poly I:C, but not LPS (personal communication, Stephen Preston). An explanation for the latter phenomenon might be that macrophages and DC may transduce the signals from the recognition of LPS by TLR4 through different adaptor molecules, which might potentially be involved in downstream effects of Japanin. Given the apparent activity of Japanin in mouse BMM, we hypothesised that Japanin modulated BMM by binding to CD206. Therefore we would expect no, or only very limited, activity of Japanin in CD206 KO BMM, as well as WT BMDC and CD206 KO BMDC, as these cells expressed CD206 only at very low levels. This hypothesis was based on the assumption that the receptor which had been shown to bind Japanin also mediated

its modulatory functions. In order to test this hypothesis, we carried out activity assays to compare the responses of WT and CD206 KO BMM and BMDC to poly I:C in the presence or absence of Japanin. However, Japanin did not cause a decrease in the expression of CD86 or TNF- $\alpha$  in poly I:C-stimulated WT BMM in our experiments; neither in WT BMDC or in the respective CD206 KO cells (Figure 23). Hence, it was not possible to draw unambiguous conclusions about the role of CD206 in the mode of action of Japanin from this experiment. We could only conclude that the expression of CD206 alone was not sufficient to make mouse BMM susceptible for modulation by Japanin. This was a surprise since the binding of Japanin to these cells appeared to be mediated by CD206.



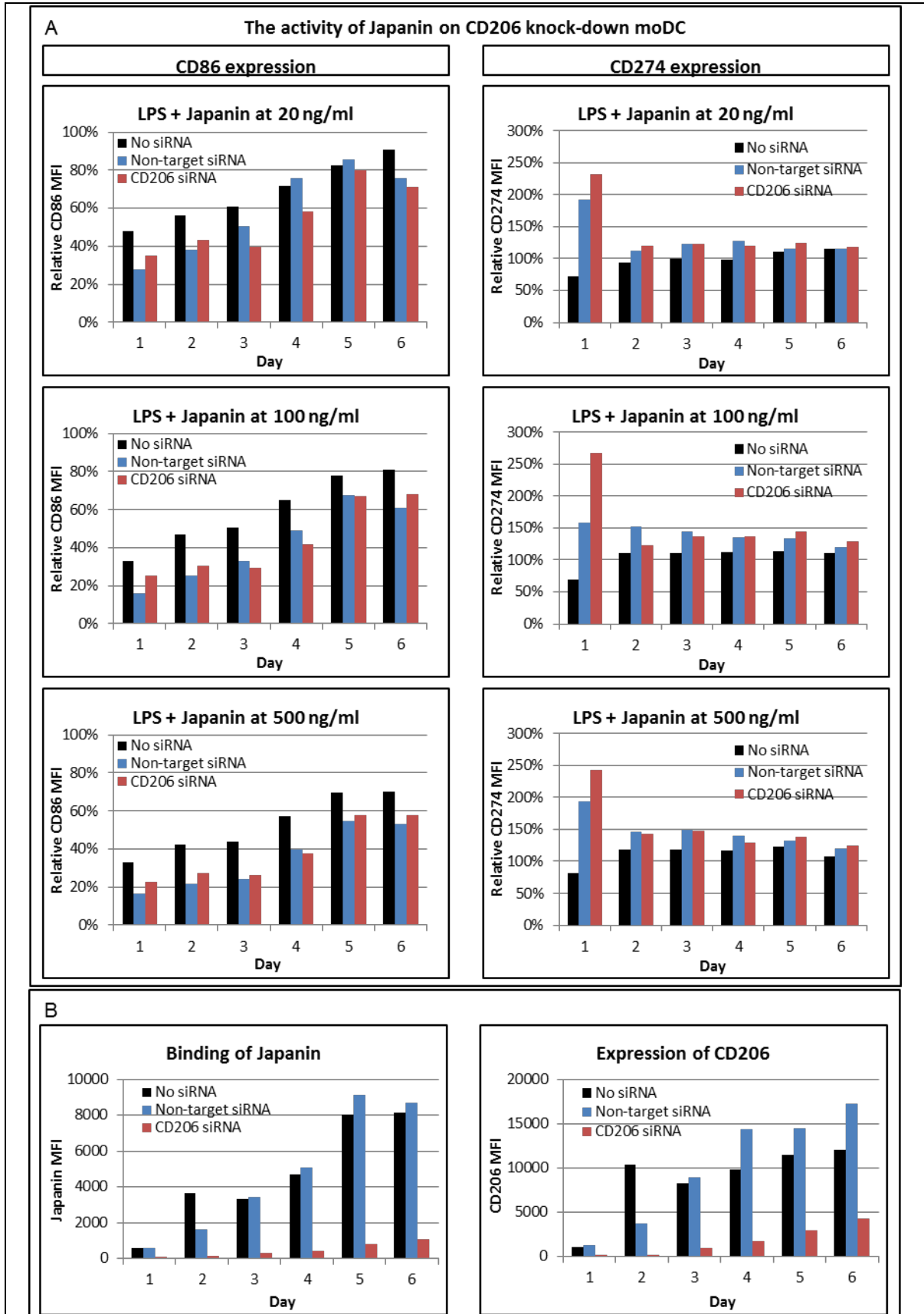
**Figure 23: The activity of Japanin on mouse BMM and BMDC.**

Bone marrow cells from WT or CD206 KO mice were differentiated into BMDC or BMM with GM-CSF or M-CSF, respectively. BMDC and BMM were incubated with Japanin and/or poly I:C for 18 hours. (A) The expression of CD86 was measured by flow cytometry. (B) The secretion of TNF- $\alpha$  was measured by ELISA. The

experiment was conducted twice; a representative result is shown; error bars indicate the standard error between duplicates.

### 3.3.3 Knock-down of CD206 in moDC does not influence the activity of Japanin

It was demonstrated in chapter 3.2.8 that a knock-down of CD206 in moDC drastically abolished the binding of Japanin (Figure 21 A). Therefore, we next turned to knock-down studies with CD206-specific siRNA in moDC in order to determine whether or not this correlated with the modulatory activity of Japanin. We hypothesised that diminished expression of CD206 would result in less modulation of LPS-induced CD86 and CD274 expression by Japanin, if the activity of Japanin was mediated by CD206. In order to test this hypothesis we set up activity assays on each day following the siRNA treatment of the moDC differentiation culture. Surprisingly, there was no significant difference in the activity of Japanin on CD206 knock-down moDC and those expressing normal levels of CD206; although also sub-optimal doses of Japanin were tested in addition to the standard dose of 500 ng/ml (Figure 24).



**Figure 24: The activity of Japanin on CD206 knock-down moDC.**

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**Figure 24: The activity of Japanin on CD206 knock-down moDC.**

Human monocytes were isolated from PBMC and immediately transfected by electroporation with CD206-specific siRNA (red). As negative controls, electroporation was also performed with non-targeting siRNA (blue), or monocytes were not electroporated nor siRNA treated (black). Differentiation into moDC was induced by IL-4 and GM-CSF in the culture medium. From day 1 of the culture, a sample of cells was taken daily to test (A) the activity of different concentrations of Japanin (20, 100, 500 ng) in the presence of LPS (100 ng/ml). Therefore the expression of CD86 and CD274 was monitored by flow cytometry. The data from each day are normalised to the respective LPS only control, i.e. no Japanin marking 100%. A no stimulus control was included in the experiment but is not shown. The experiment was performed once. (B) For reference, the binding of Japanin and the expression of CD206 from Figure 21 A are depicted for each day as MFI, subtractive of OmCI and isotype controls.

If only few receptors were needed to trigger a response to Japanin, the reduced expression of CD206 might still have been enough to mediate the activity of Japanin, since the receptor was not completely eliminated by the knock-down. Still, we would expect a difference in the efficacy of Japanin when applied at suboptimal doses, which we did not see. Hence, the results strongly suggest that CD206 is not essential for the mode of action of Japanin, even though this receptor appears to be responsible for at least much of the binding of Japanin to moDC that we could detect (Figure 21). This lead to the question as to whether the observed binding of Japanin was at all necessary for its activity and, in turn, if cells that do not appear to bind Japanin might still respond to it.

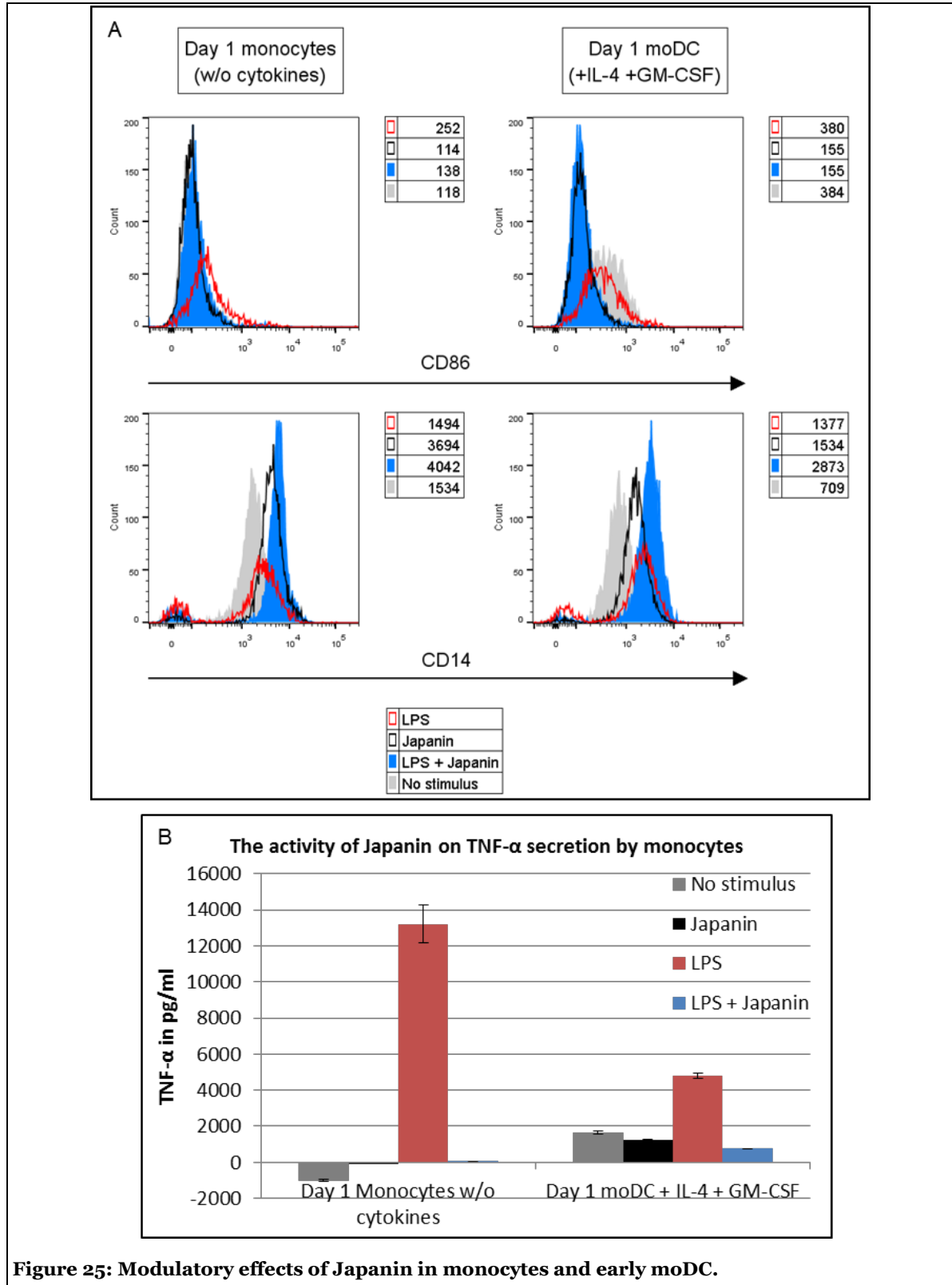
#### **3.3.4 Monocytes can be modulated by Japanin**

Trying to find a correlation between the binding and the activity of Japanin, we raised the question whether functions of monocytes, on which no expression of CD206 or binding of Japanin had been detected, could be modulated by Japanin. We already knew that Japanin prevented the differentiation of monocytes into moDC, as concluded from the sustained expression of CD14 and inhibited expression of CD1a on moDC generated in the presence of Japanin (Figure 6). However, we had reasoned that this modulation was enabled by upregulation of the Japanin-binding receptor, which appears to be CD206,

during the culture. Now we aimed to test whether Japanin modulated cultured monocytes in the absence of DC-polarising cytokines, which would most likely not acquire the expression of CD206. Monocytes were cultured no longer than 18 hours after isolation in the presence of Japanin, LPS, both, or no stimulus. The expression of CD86 and CD14 as well as the secretion of TNF- $\alpha$  were measured and compared to a monocyte culture that was set up in parallel in the presence of IL-4 and GM-CSF to represent early differentiated moDC as a reference. We found that monocytes cultured in the absence of cytokines could be modulated by Japanin, as could early moDC. Japanin prevented the upregulation of LPS-induced expression of CD86 equally well in monocytes and early moDC (Figure 25 A). Japanin also promoted the expression of CD14 in monocytes and early moDC, both in the presence and absence of LPS. Meanwhile an overall lower expression of CD14 in the cultures with IL-4 and GM-CSF indicated that these cells had begun to differentiate into moDC, while monocytes cultured in the absence of cytokines had not. Moreover, Japanin strongly inhibited the secretion of TNF- $\alpha$  by monocytes and early moDC (Figure 25 B). The effect appeared even more pronounced in monocytes, as they responded to LPS with higher concentrations of TNF- $\alpha$  than early moDC and Japanin almost completely abolished secretion of the cytokine by both cell types. These results suggested that CD206 was not required for the mode of action of Japanin in the modulation of monocytes. Notably, the results also suggested that only low intensity binding of Japanin to monocytes was required for the modulatory activity of Japanin in these cells, as the binding of Japanin to monocytes correlated with the expression of CD206 and was almost undetectable (Figure 12, Figure 21 A). Yet we could not exclude that monocytes had partly differentiated during the 18 hour culture in the absence of IL-4 and CM-CSF, for example

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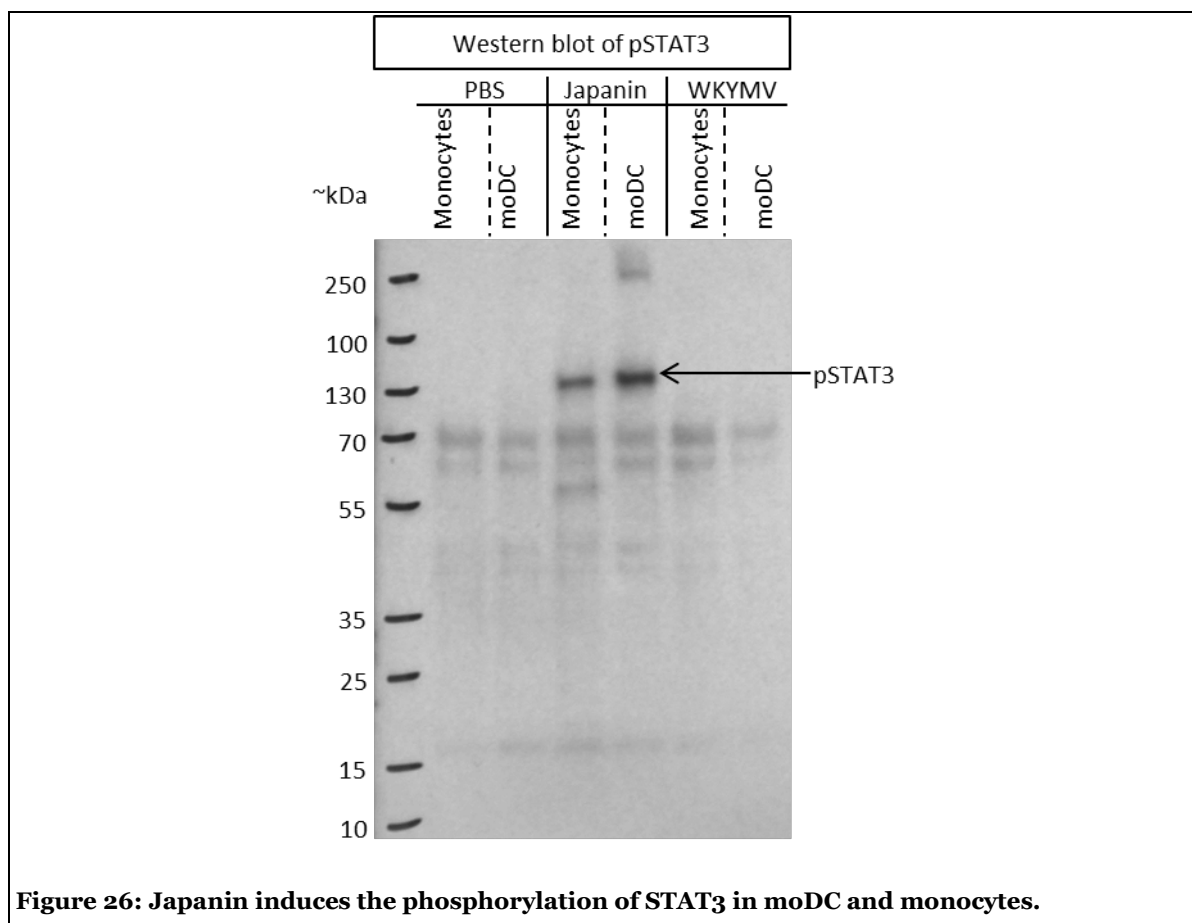
induced by contact with the culture surface, which might have led to the expression of receptors that are not usually expressed by monocytes.



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Monocytes were cultured either without cytokines or with IL-4 and GM-CSF for differentiation into moDC. Cells were stimulated from the beginning of the culture with Japanin and/or LPS. After an 18 hour incubation, (A) cells were stained with fluorescently labelled antibodies to measure the expression of CD86 and CD14 by flow cytometry. Boxes to the right of each histogram indicate the respective MFI. (B) Cell culture supernatant was analysed for TNF- $\alpha$  by ELISA. The experiment was performed in triplicates with monocytes from one blood donor; error bars indicate the standard error.

We wished to verify the activity of Japanin in monocytes by an assay that would exclude the chance of spontaneous differentiation during the relatively long culture of 18 hours. Previous experiments with moDC in our lab had shown that the phosphorylation of STAT3 could be induced by Japanin within minutes upon administration. Hence, we performed this signalling assay with freshly isolated monocytes in comparison to completely differentiated moDC. It was found that the phosphorylation of STAT3 was induced by Japanin not only in moDC, but also in monocytes (Figure 26).



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Monocytes or moDC were incubated with Japanin at 5 µg/ml for 7 min. PBS and an irrelevant peptide (WKYMV) were used as negative controls. Cells were harvested in chilled PBS, pelleted, lysed, and snap-frozen. Thawed cell lysates were analysed by SDS PAGE and subsequent western blotting with an antibody against phosphorylated STAT3 (pSTAT3).

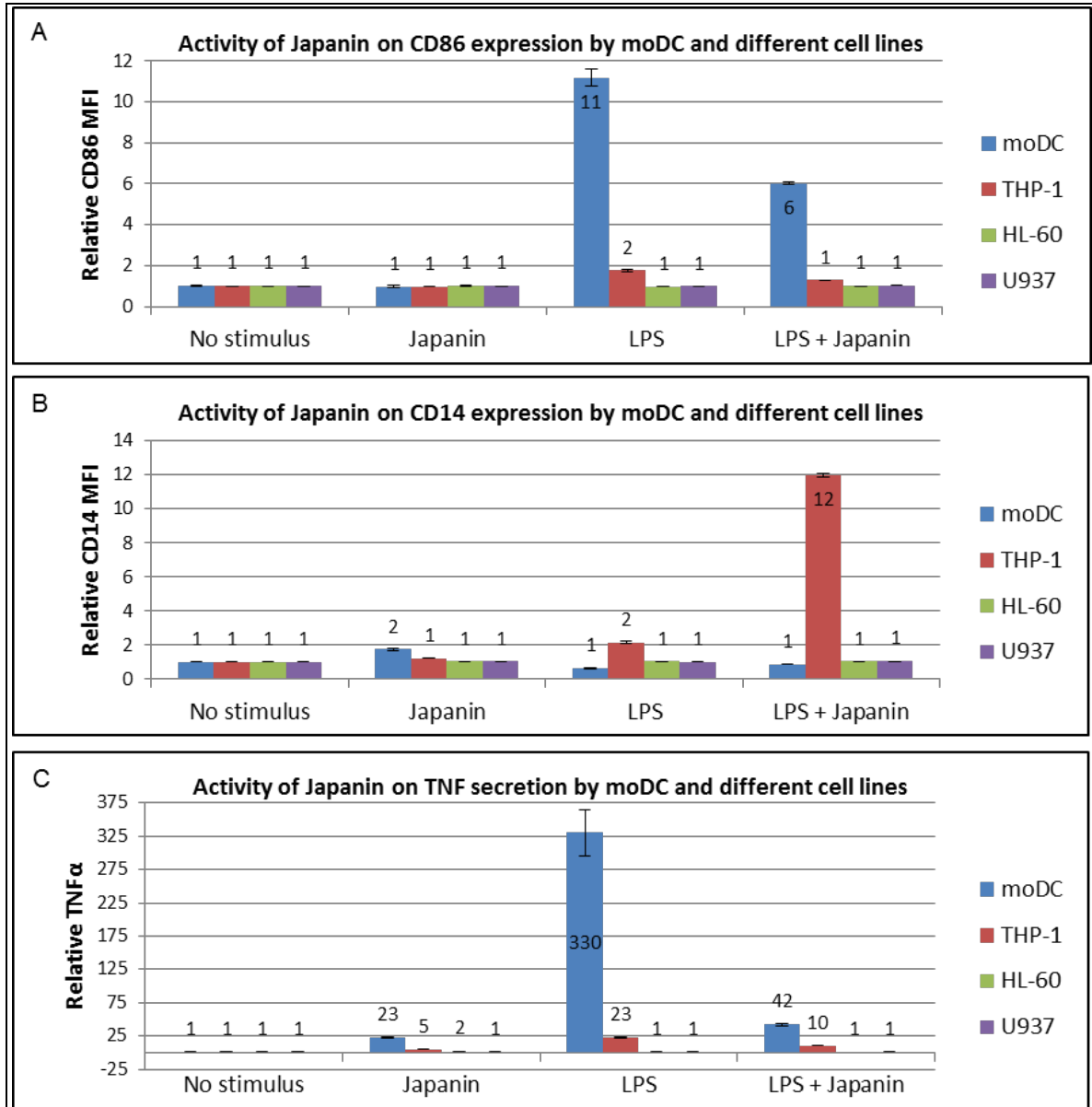
These results demonstrated that Japanin could modulate cells which lack expression of CD206 or measurable binding of Japanin. This strongly suggests that the expression of CD206 is not necessary for the activity of Japanin, despite the finding that this receptor mediates binding of Japanin to moDC. Apparently the activity of Japanin is conferred in a different manner that requires only a very low level or a very short time of binding. We might not have been able to detect more binding of Japanin to CD206-negative cells due to limitations of our methods.

### 3.3.5 Japanin modulates stimulated THP-1 cells

The finding that lack of the expression of CD206 or measurable binding of Japanin did not exclude modulatory activity of Japanin on monocytes was surprising. However, in retrospect we saw new potential in the cell lines we screened for the binding of Japanin in our earlier receptor identification studies (Figure 13, Figure 14). Since none of them expressed CD206 or bound Japanin to a detectable extent, we had not previously tested the activity of Japanin on them. Now, having shown modulatory functions of Japanin in human monocytes, we tested the response of our human monocytic cell lines to Japanin. The cells were incubated with Japanin, LPS, both, or no stimulus and CD86, CD14 and TNF-α were measured after 18 hours of culture. In response to LPS, THP-1 cells increased expression of CD86 and CD14 as well as the secretion of TNF-α, whereas HL-60 and U937 did not respond to the stimulus (Figure 27). The LPS-induced upregulation of CD86 and TNF-α by THP-1 cells was inhibited by Japanin (Figure 27 A+C), whilst the expression of

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CD14 was greatly enhanced in the presence of Japanin (Figure 27 B), similar as seen in monocytes and moDC.



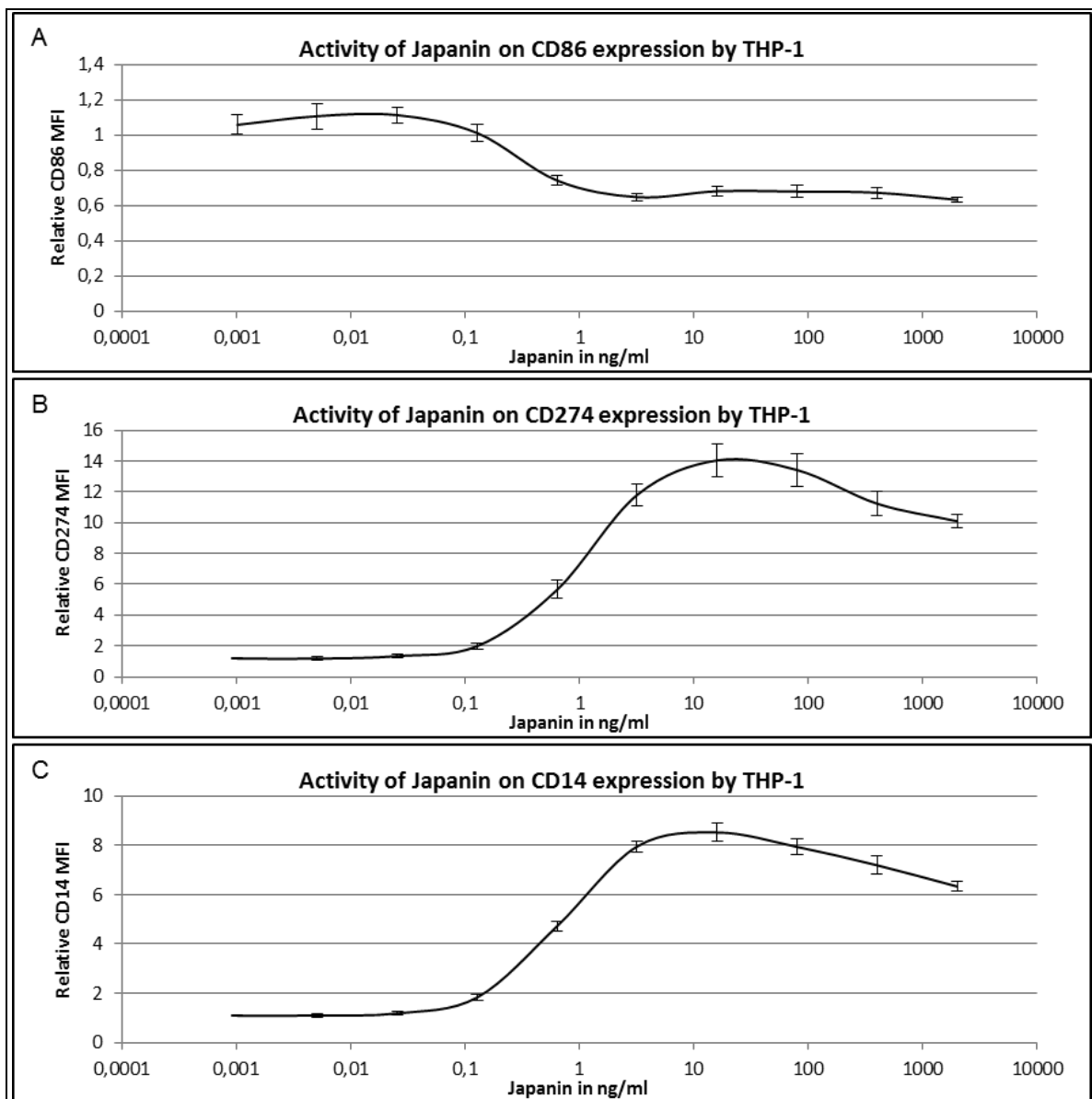
**Figure 27: The response of THP-1 cells to LPS is modulated by Japanin.**

MoDC and cell lines (THP-1, HL-60, U937) were stimulated with LPS in the presence or absence of Japanin for 18 hours. At the end of the incubation time the cells were stained with fluorescently labelled antibodies to measure surface molecule expression of (A) CD86 and (B) CD14 by flow cytometry. (C) Cell culture supernatants were analysed for TNF- $\alpha$  secretion by ELISA. Values are normalised to the no stimulus control as 1. Error bars indicate the standard error between triplicates of one experiment.

Since monocytes and THP-1 cells did not appear to express CD206 and did not bind detectable amounts of Japanin but could be modulated by Japanin, we hypothesised that the binding of Japanin to CD206 was due solely to the scavenging function of CD206 and the glycosylation of Japanin. If this were true, cells which did not express CD206 might be more sensitive to Japanin than those that expressed CD206, as the scavenging by CD206 might hinder Japanin from binding to the actual activity-conferring receptor. We had previously determined a dose-response curve of Japanin on LPS-stimulated moDC, which greatly express CD206 and bind Japanin. The maximal effective dose of Japanin on moDC was on average >1000 ng/ml (Figure 5). In order to compare the dose response of THP-1 cells and moDC, we measured decrease or increase in the expression of CD86, CD274, and CD14 by LPS-treated THP-1 cells in the presence of graded doses of Japanin. An increasing concentration of Japanin correlated with a reduced expression of CD86 and an enhanced expression of CD274 as well as CD14 by THP-1 cells (Figure 28). Yet the maximal effective dose was reached at approximately 10 ng/ml; so it appeared THP-1 cells were about 100 times more sensitive to Japanin than moDC. This increased sensitivity to Japanin could be due to the lack of CD206, suggesting that Japanin was no longer being scavenged in the absence of CD206. Since the binding of Japanin to CD206 appears to be glycan-dependent (Figure 17, Figure 19), it would have been interesting to confirm this hypothesis by treating CD206-expressing moDC with non-glycosylated Japanin. The lack of glycosylation might have increased the efficacy of Japanin on moDC, whereas it should not change the dose-response of THP-1. As explained earlier (3.2.6), we have been unable to produce pure and stable preparations of non-glycosylated Japanin as glycosylation appeared to be essential for the stability of the protein. However, the activity assays with CD206 knock-out moDC suggested that the presence of CD206 did not reduce the activity

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of Japanin on moDC, as there was no significant correlation between the efficacy of Japanin and the expression of CD206 (Figure 24). On the other hand the efficacy of Japanin generally appeared to decrease during the course of the differentiation of moDC, regardless of the expression of CD206. This might suggest that Japanin has a higher efficacy in monocytes than in moDC, yet most likely due to factors other than CD206.



**Figure 28: The activity of Japanin in THP-1 cells is dose-dependent.**

THP-1 cells were stimulated with LPS and graded doses of Japanin for 18 hours. At the end of the incubation the cells were stained with fluorescently labelled antibodies to measure surface molecule expression of (A) CD86, (B) CD274, or (C) CD14 by flow cytometry. Values are normalised to no stimulus (equals null) and LPS only (equals 1) controls. Error bars indicate the standard error between triplicates in one experiment.

### 3.3.6 Attempts to enhance the binding of Japanin to THP-1 cells

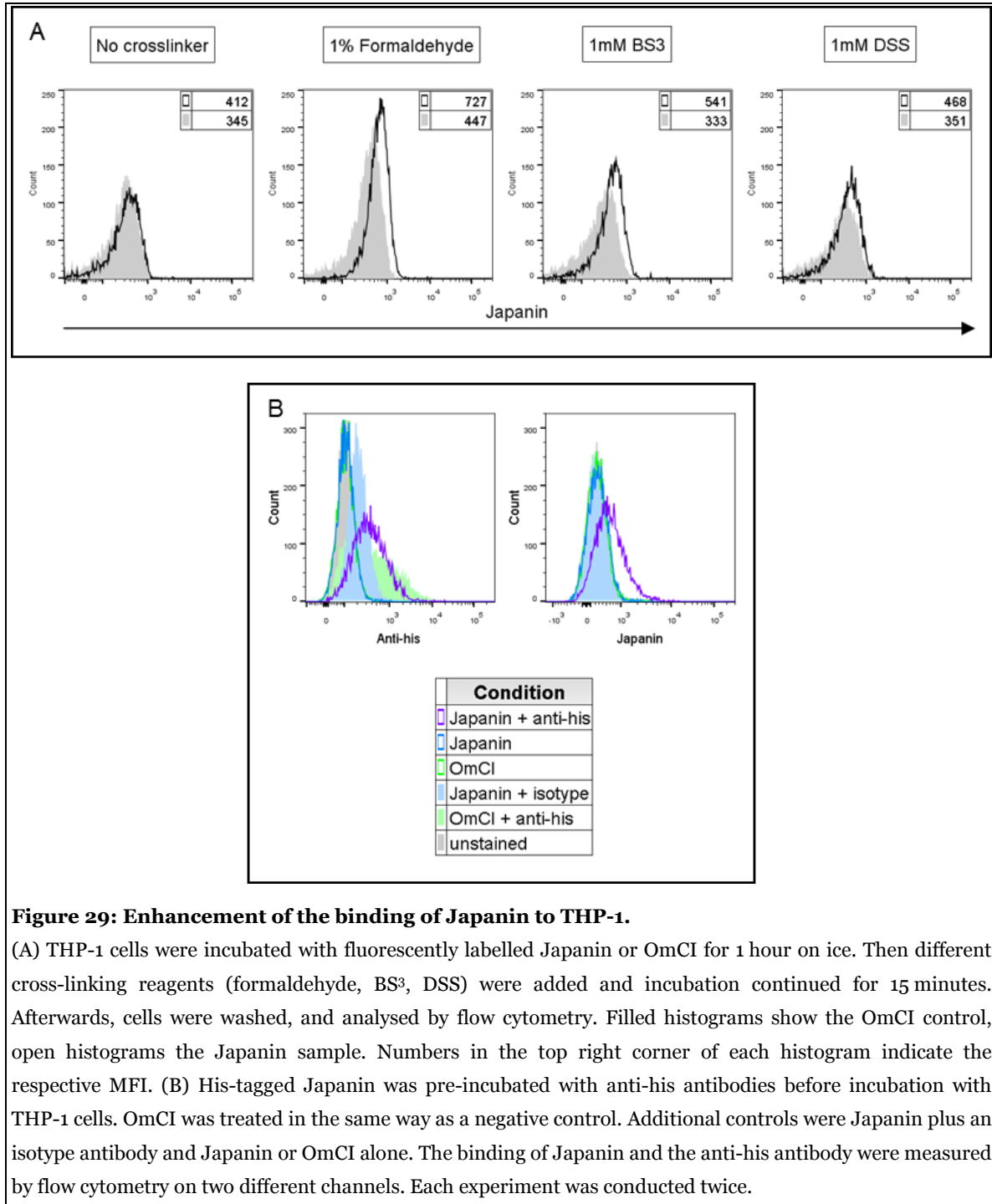
Since the activity of Japanin on monocytes and THP-1 cells was comparable to the modulatory functions on moDC, but only the latter cell type expressed CD206, we hypothesised that there may be other receptors on these cells that mediated the effects of Japanin. These receptors would presumably be expressed at very low levels, or might bind Japanin with only very low affinity. To endeavour to identify these receptors, we attempted to enhance the binding of Japanin to THP-1 cells, potentially enabling further investigations.

Firstly, we tried to stabilise the binding of Japanin to THP-1 cells with cross-linking reagents. The level of detectable binding increased, although not much, when THP-1 cells and Japanin were incubated together in the presence of cross-linkers (Figure 29 A). This suggested that there may be only weak interactions between Japanin and putative receptors. Compared to the binding of Japanin in our previous pull-down experiment, which identified CD206 as a Japanin-binding receptor (Figure 15 C), this approach did not seem likely to allow pull-down of detectable amounts of cross-linked Japanin-receptor complexes from THP-1 cells.

Secondly, we attempted to increase the affinity of the binding to THP-1 cells with polymers of Japanin. Therefore, his-tagged Japanin was incubated with anti-his tag antibodies which could in theory link multiple Japanin molecules together. The binding of these Japanin polymers did indeed result in an increased signal, however, so did the binding of the antibodies alone (Figure 29 B). Hence, it appeared that polymerisation of Japanin did not actually increase the binding of Japanin to THP-1 cells. Instead, the apparent increase in the binding of Japanin was most likely a result from the binding of antibody-bound

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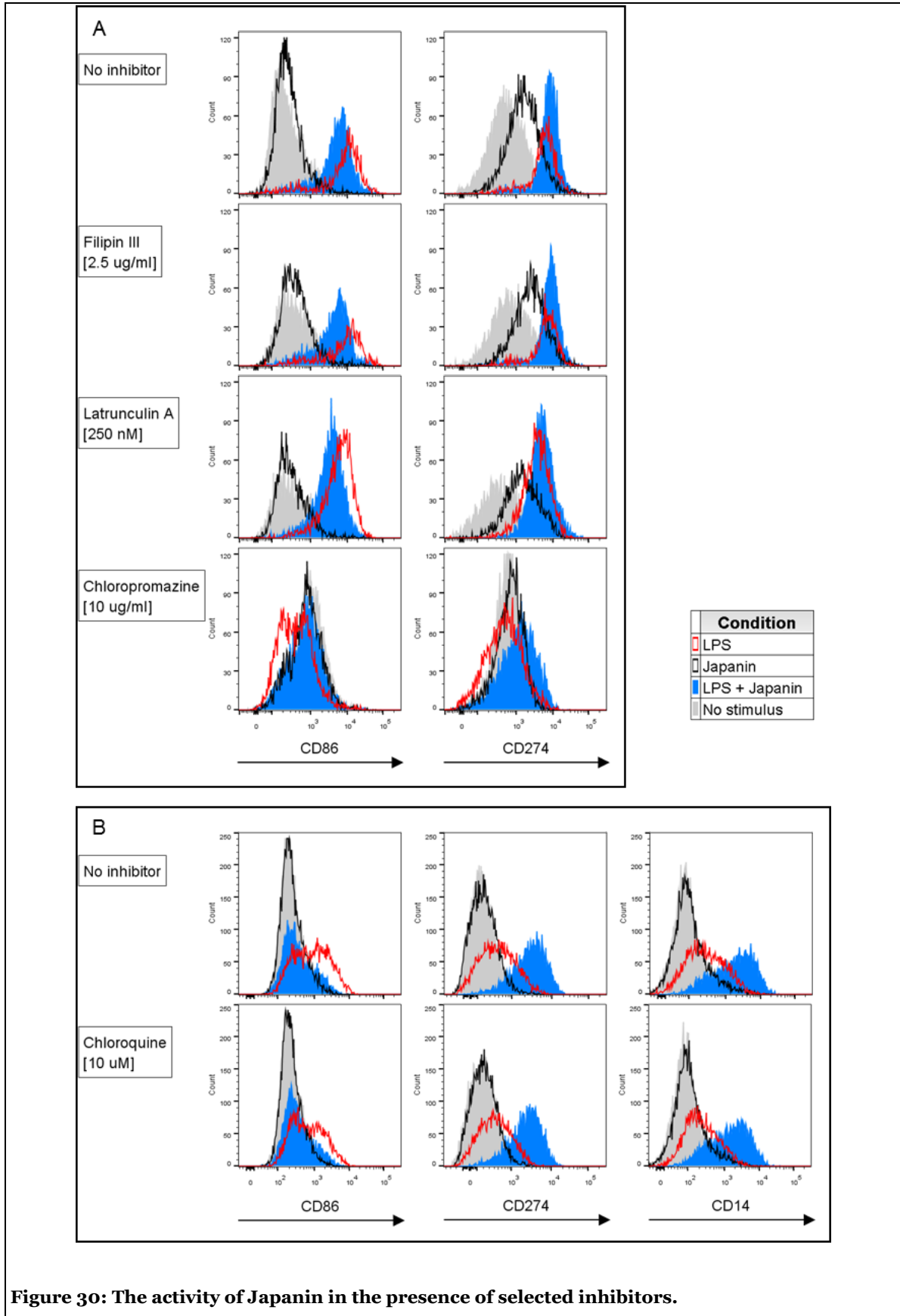
Japanin to Fc receptors on THP-1 cells; despite attempts to block Fc receptors with mouse serum.



### 3.3.7 Endocytosis inhibitors do not interfere with the effects of Japanin

Previous studies in our lab had shown that Japanin was internalised by moDC and localised in endosomes (personal communication, Oliwia Rysnik). This observation is consistent with our finding that the binding of Japanin to moDC is mediated by CD206, since it traffics between endosomes and the cell surface as a recycling receptor. However, we also found that the expression of CD206 is not required for the modulatory functions of Japanin. Hence it might be possible that the internalisation was only a consequence of the binding to CD206, but not actually part of the mode of action of Japanin. Alternatively, Japanin might bind to an internal receptor and therefore needed to be taken up by endocytosis, which may be mediated by CD206 but also by other receptors. To test this possibility, we measured the activity of Japanin in the presence of selected endocytosis inhibitors: Filipin III has been reported to bind cholesterol and inhibit lipid raft- or caveolae-dependent endocytosis, latrunculin A and chlorpromazine have been reported to prevent polymerisation of actin and inhibit clathrin-dependent endocytosis, and chloroquine has been reported to inhibit acidification of endosomes, affecting their sorting functions. In an initial screen, none of these inhibitors impaired the activity of Japanin (Figure 30). Besides, chlorpromazine completely blocked the upregulation of CD86 and CD274 in response to LPS, so that in turn no Japanin-induced decrease could be measured. The results might suggest that endocytosis may not be required for the activity of Japanin, yet it cannot be excluded from this experiment that internalisation inside the cell is required for the mode of action of Japanin.

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**Figure 30: The activity of Japanin in the presence of selected inhibitors.**

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(A) MoDC or (B) THP-1 cells were cultured for 18 hours in the presence or absence of Japanin and/or LPS, with and without addition of endocytosis inhibitors. Expression of CD86, CD274 and (B) CD14 was measured by flow cytometry. Representative histograms from triplicates are shown.

### 3.3.8 Summary of chapter 3.3

Having identified CD206 as a Japanin-binding receptor on moDC, we investigated its role in the modulatory functions of Japanin. It was found that the activity of Japanin on moDC was not hindered by the presence of soluble CD206 constructs, whilst this had been shown to reduce the binding. Likewise, the knock-down of CD206 in moDC did not impair the activity of Japanin, suggesting that CD206 had no major function in the mode of action of Japanin. On the contrary, the efficacy of Japanin appeared higher in early moDC and the monocytic cell line THP-1 compared to fully differentiated moDC. Interestingly, although the binding of Japanin to monocytes and THP-1 cells was almost unobservable, which suggested that the functions of Japanin were mediated by receptors which were expressed at very low frequency or which bound with very low affinity, or that the activity of Japanin was conferred by an internal receptor. However, selected endocytosis inhibitors did not affect the activity of Japanin, which might suggest that endocytosis – whether mediated by CD206 or another receptor - might not be required for the mode of action of Japanin. Yet we cannot exclude that internalisation inside the cell is generally involved in the mechanism of action of Japanin.

### 3.4 Potential CD206-independent mechanisms of action of Japanin

The results of the previous chapter suggested that the expression of CD206 was dispensable for the activity of Japanin, and so appeared to be much of the binding of Japanin to cells. Therefore, this chapter aims to investigate alternative potential modes of action of Japanin that would not involve CD206.

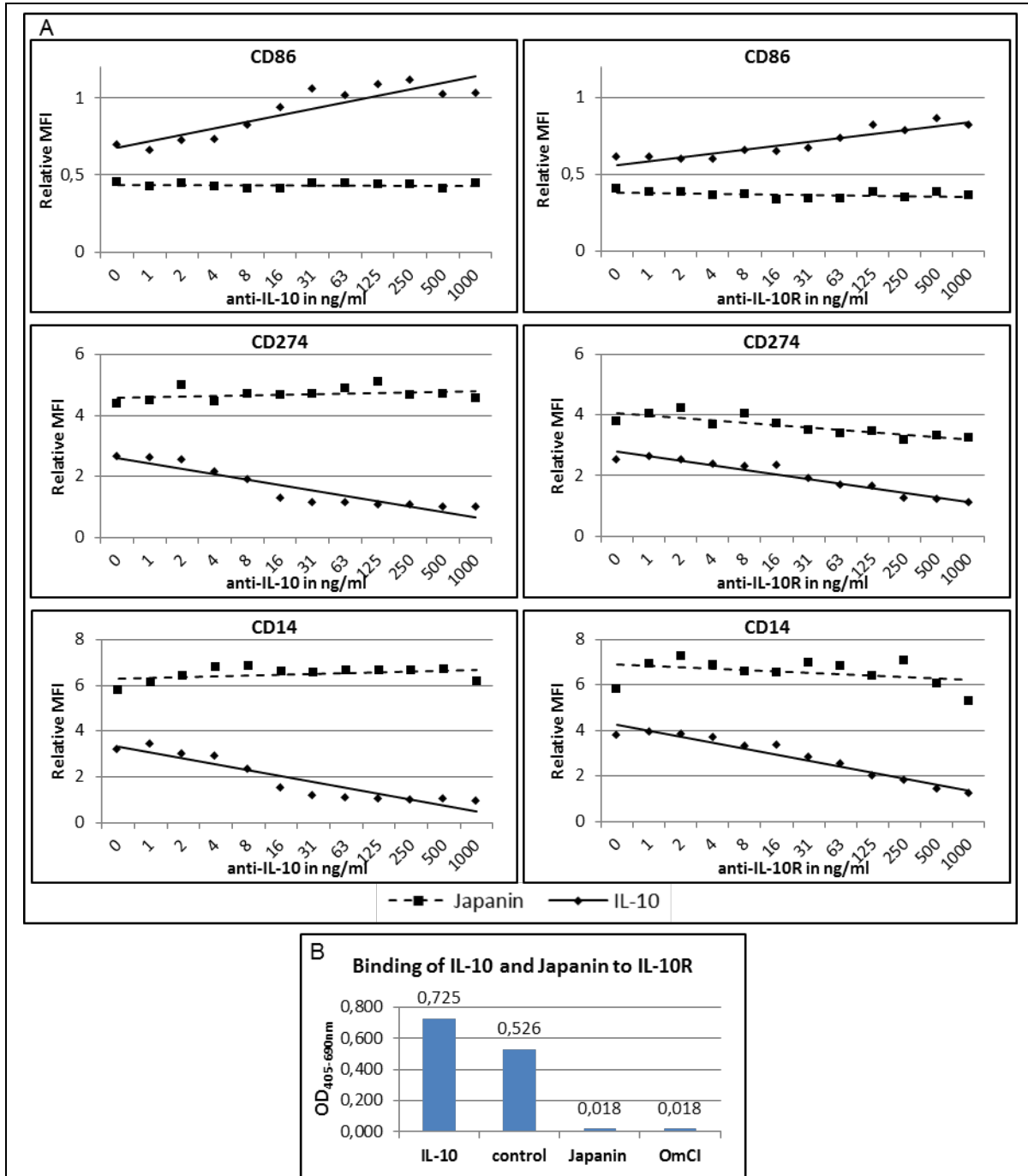
#### 3.4.1 The effects of Japanin are not mediated by IL-10

The modulatory functions of Japanin described in chapter 3.1 show great similarities with IL-10. Since Japanin induced the expression of IL-10 in moDC, we hypothesised that the other activities of Japanin might be downstream effects of Japanin-induced IL-10. Alternatively, due to structural similarities, such as similar size and formation of dimers, it could be hypothesised that Japanin might mimic IL-10 and might bind to the IL-10 receptor (IL-10R). In order to test these hypotheses, investigations were undertaken into whether the activity of Japanin depended on IL-10 or its receptor. Therefore, we measured the activity of Japanin in the presence of graded doses of anti-IL-10 or anti-IL-10R antibodies by the expression of CD86, CD274 and CD14 in LPS-treated THP-1 cells (Figure 31 A). As a positive control for the blocking capacity of the antibodies, we also tested the activity of recombinant IL-10 under the same conditions. LPS-induced expression of CD86 was significantly decreased by Japanin and IL-10, while the expression of CD274 and CD14 was greatly upregulated. The effects of IL-10 were gradually inhibited with increasing concentrations of anti-IL-10 and anti-IL10R antibodies, verifying that these antibodies could inhibit the effects of IL-10. On the contrary, the CD86-reducing function of Japanin was not inhibited by either antibody, suggesting that this activity is IL-10 independent. However, the Japanin-induced upregulation of CD274 and CD14, showed a

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slight decrease with increasing doses of anti-IL-10R but not anti-IL-10 antibodies. We therefore wished to investigate whether Japanin bound directly to the IL-10R. Recombinant IL-10R was immobilised on a microtitre plate and incubated with biotinylated Japanin, which enabled photometric detection after addition of a specific substrate for streptavidin-conjugated peroxidase (Figure 31 B). As a control for unspecific binding, OmCI was incubated under the same conditions. Successful immobilisation of IL-10R was confirmed by application of recombinant IL-10, the binding of which was detected by anti-IL-10 antibodies plus a peroxidase-conjugated secondary antibody. We did not detect any binding of Japanin to the IL-10R in this assay. Hence, we hypothesised that the modulatory functions of Japanin were mediated by a receptor other than IL-10R, which is yet to be found.



**Figure 31: The activity of Japanin in the presence of anti-IL-10/R.**

(A) THP-1 cells were cultured for 18 hours with graded doses of anti-IL-10 or anti-IL-10R antibodies in the presence of LPS and Japanin (1 ng/ml) or IL-10 (10 ng/ml). The expression of CD86, CD274 and CD14 was measured by flow cytometry. MFI values are normalised to the LPS only control, i.e. no Japanin or IL-10 marking 1. The experiment was conducted >3 times with different concentrations of Japanin and IL-10. Representative graphs are shown; lines indicate the linear trend. (B) Recombinant human IL-10 receptors were immobilised on a micro titre plate and incubated with recombinant IL-10 or biotinylated Japanin. No ligand was used as a negative control for IL-10 and biotinylated OmCI as a negative control for Japanin. IL-10 was detected with anti-IL-10 antibodies plus a peroxidase-conjugated secondary antibody; both were also

added to the control. Japanin and OmCI were detected with peroxidase-conjugated streptavidin. Only one experiment was conducted due to limited obtainability of recombinant IL-10 and IL-10 receptors.

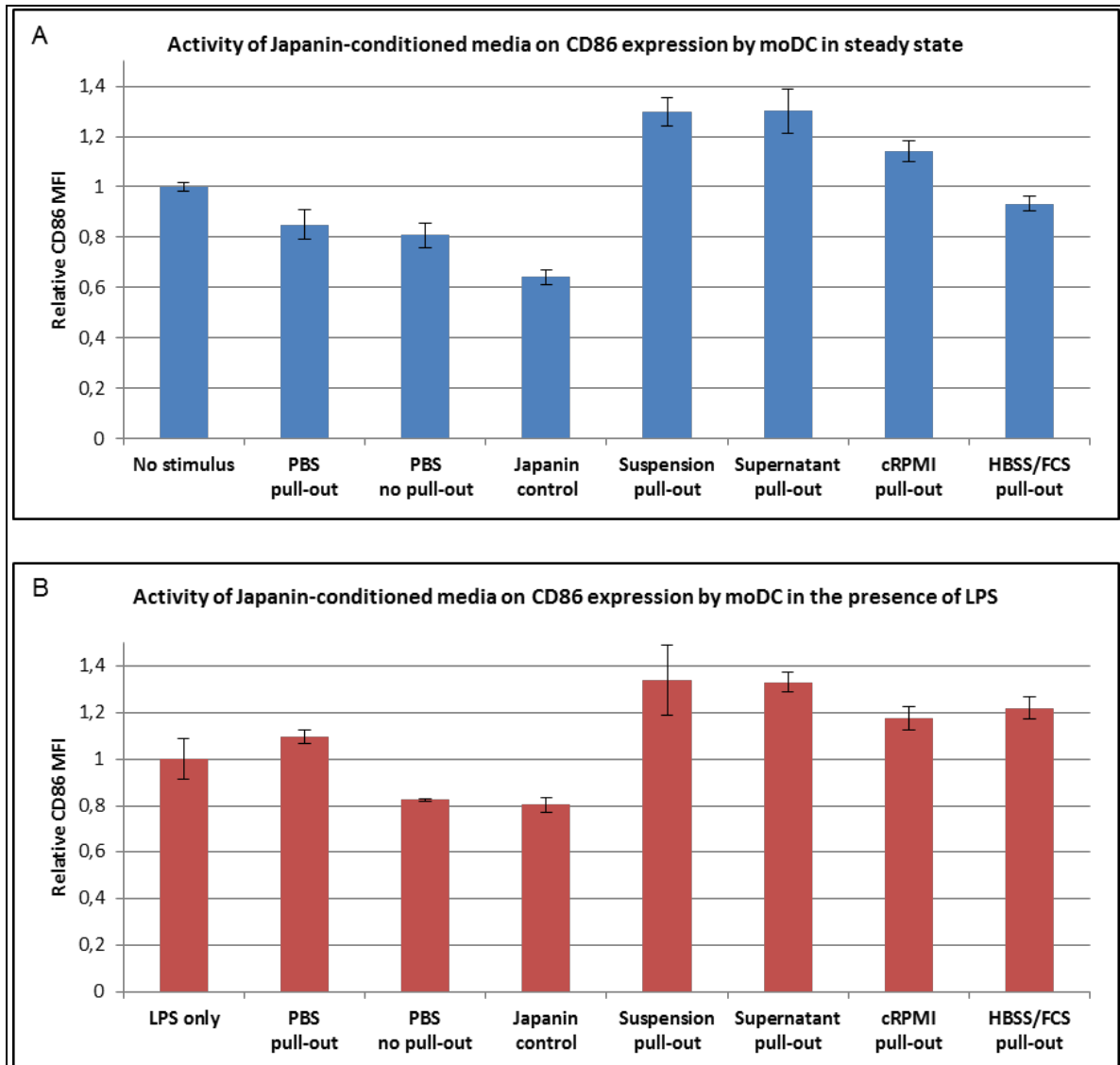
### 3.4.2 The activity of Japanin requires cell contact

Prostaglandins, such as PGD<sub>2</sub> or PGE<sub>2</sub> have been shown to promote tolerogenic DC maturation and thereby promote polarisation of Treg cells <sup>144</sup>. The production of these prostaglandins may be catalysed by enzymatic activity of lipocalins, such as L-PGDS <sup>254,256</sup>. Therefore we hypothesised that Japanin might have enzymatic activity and may synthesise an immunoactive compound, which was responsible for what we observed as the activity of Japanin. Since the binding of Japanin to monocytes and THP-1 cells appeared to be almost undetectable but Japanin showed activity on these cells, we hypothesised that Japanin might exert this function by catalysing components in the culture medium and would therefore not require direct binding to cells via receptors. In order to test this hypothesis, investigations were made as to whether Japanin-conditioned medium, containing the putative active product, would have modulatory effects on DC. Different medium compositions were set up in order to narrow down possible factors that could serve Japanin as a substrate. We speculated a potential substrate might be a cell-derived component, an ingredient in basal medium, or a serum factor. In order to test these compounds, Japanin was incubated overnight with THP-1 cells, THP-1 supernatant, complete RPMI (with L-glutamine and FCS), HBSS containing 2% FCS (HBSS/FCS), and PBS. The PBS condition represented a negative control, with no putative substrate for Japanin. After a 24 hour incubation at 37°C, Japanin was removed from each condition by using magnetic TALON™ beads that would bind Japanin by its his tag. Half of the PBS condition was set aside before this step, so that Japanin was not removed by TALON™ beads. This was used as a control for the stability of Japanin over the 24 hour incubation

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at 37 °C. In order to test the activity of Japanin-conditioned media, each condition was mixed in a 1:1 ratio with moDC suspensions in complete RPMI and incubated for 18 hours at 37°C. As a control for the usual activity of Japanin, freshly diluted Japanin was also applied, as well as fresh PBS as a 'no stimulus' control. The activity of Japanin was tested in the absence and presence of LPS, assessed by the expression of CD86 by moDC. None of the Japanin-conditioned media significantly reduced the expression of CD86 when compared to the 'no stimulus' control (Figure 32). Upon stimulation with LPS, the Japanin stability control, that is Japanin conditioned in PBS without TALON™ pull-out (PBS no pull-out), decreased the expression of CD86 as much as freshly diluted Japanin, whilst the PBS negative control (PBS pull-out) did not (Figure 32 B). This showed that Japanin had been stable during the incubation and that the pull-out of Japanin from the conditions with TALON™ beads had been successful. We conclude from this experiment that the activity of Japanin most likely requires cell contact. We do not exclude the possibility that Japanin may act as an enzyme, as it might exert this function after internalisation inside the cell. Therefore we propose that this may be a potential mechanism of action of Japanin that would be interesting to investigate in future studies.



**Figure 32: Activity of Japanin-conditioned medium.**

Different conditions were incubated with Japanin for 24 hours: THP-1 cells, THP-1 culture supernatant, complete RPMI, HBSS/FCS, PBS. Japanin was pulled-out of the media with magnetic TALON™ beads, binding the his tag. Japanin incubated in PBS that underwent subsequent TALON™ pull-out served as a negative control, while Japanin incubated in PBS without TALON™ pull-out was a stability control. Additionally, freshly diluted Japanin, i.e. not pre-incubated for 24 hours was a further positive control. Conditioned media or freshly diluted Japanin were added 1:1 to moDC suspension and incubated for 18 hours in the (B) presence or (A) absence of LPS. The expression of CD86 was measured by flow cytometry. The experiment was performed twice, except for the PBS no pull-out control, which was only included in one experiment. Values are normalised to (A) no stimulus, or (B) LPS only. Error bars indicate the standard error.

### 3.4.3 Summary of chapter 3.4

Based on the modulatory functions of Japanin, we hypothesised that these might be mediated by the induction of IL-10, or direct binding of Japanin to its receptor. However, blocking experiments with specific antibodies and binding assays with recombinant receptors could not prove this hypothesis, suggesting that the mode of action of Japanin is IL-10 independent. We also hypothesised that Japanin might have enzymatic properties and may produce an immunoactive compound; a mechanism that might potentially not require cell contact. Therefore we undertook experiments with Japanin-conditioned media. Yet these experiments could not confirm our hypothesis and suggested that direct contact with cells may be needed in order to mediate the modulatory functions of Japanin. Cell contact might result in the internalisation of Japanin inside the cell, potentially enabling interactions with intracellular receptors.

## 4 Discussion

### 4.1 Modulatory functions of Japanin

#### 4.1.1 Possible use for Japanin in ticks

Since it takes hard ticks several days to complete a blood meal, their survival depends on successful strategies to avoid destruction by immune defence mechanisms in the host. These strategies may include components in tick saliva, as SGE from many different tick species contain compounds that act on host immune cells and humoral mediators, therefore potentially facilitating tick survival. We hypothesised that metastriate ticks, such as *R. appendiculatus*, might use Japanin, or Japanin-like lipocalins, as a strategy to evade elimination by the host immune system <sup>290</sup>. Given that homologue Japanin proteins are found exclusively in metastriate but not in prostriate ticks, Japanin appears to be the first identified member of a distinct family of DC-specific modulators within the ixodid ticks. DC-modulatory proteins have also been described in prostriate ixodid species, but none of them acts strictly on DC. Salp15 for example binds to DC-SIGN on DC, inhibits their pro-inflammatory cytokine production and prevents the activation of T cells. However, in contrast to Japanin, Salp15 has also direct inhibitory effects on T helper cells by binding to the CD4 co-receptor. Salp15 homologues have been identified in other prostriate, but not in metastriate, ticks. Thus it appears that Japanin- and Salp15-like immunomodulators may have evolved in different lineages of ixodid ticks as convergent mechanisms to subvert host defence. Moreover, we found that the immunosuppressive functions of Japanin encompassed a wide spectrum of stimuli. This may be advantageous for ticks, since it might protect them from eradication by the host immune defence during the

feeding process, in which potentially immunoactive components may not only be released by the tick, but also tick-borne pathogens.

#### 4.1.2 Potential role for Japanin in the transmission of tick-borne pathogens

The example of Salp15 demonstrates that immunosuppressive tick-salivary proteins may also protect tick-transmitted pathogens from host rejection, as has been shown by studies with *Borrelia burgdorferi*. Indirect protection from an immune response against *B. burgdorferi* might already be provided by the DC- and T cell-inhibiting effects of Salp15. Additionally, Salp15 has been shown to bind directly to an outer surface protein of *B. burgdorferi*, thereby preventing the binding of antibodies to the bacterium. Given that metastriate ticks, such as *R. appendiculatus*, are also vectors of pathogens, we hypothesise that Japanin might be exploited by pathogens for their transmission. The transmission mechanisms of many metastriate tick-borne pathogens are not fully understood. For example the transmission of Thogoto virus and tick-borne encephalitis (TBE) virus by *R. appendiculatus* have been associated with an as-yet-unidentified protein in SGE <sup>315</sup>. Likewise, saliva from *R. appendiculatus* appears to promote the transmission of *Theileria parva*, a protozoan parasite that causes East Coast fever in cattle, but the component responsible has not yet been identified. East Coast fever is a disease of economic importance as it constrains the livestock production in eastern Africa <sup>316</sup>. Interestingly, the transmission of *T. parva* depends around the third day of tick feeding, which is also the time point when Japanin is produced in tick salivary glands. This may indicate that Japanin might be involved in pathogen transmission. Japanin homologues have been identified in SGE from *R. sanguineus*, which feeds primarily on dogs but occasionally also on humans. A bite from *R. sanguineus* may cause Mediterranean spotted fever, as it transmits the

bacterium *Rickettsia conorii*<sup>317</sup>. Japanin-like proteins might also play a role in Rocky Mountain spotted fever, which is transmitted to humans by the Rocky Mountain wood tick *Dermacentor andersoni*, another metastriate species in which Japanin-like lipocalins have been identified.

If Japanin proteins were involved in pathogen transmission, targeting Japanin might prove valuable in preventing diseases that are mediated by metastriate ticks, for example by designing Japanin-specific vaccines. Recombinant versions of secreted tick saliva compounds from *R. appendiculatus* and other species have already been shown to represent potent vaccine antigens for tick-borne diseases in experimental animal models<sup>318</sup>. Hence, we propose that targeting Japanin with a specific vaccine might enable the host immune system to eliminate ticks and thereby eliminate tick-borne pathogens as well as. But firstly, more studies are needed in order to confirm the proposed involvement of Japanin in pathogen transmission. The role of Salp15 in the transmission of *B. burgdorferi* has been demonstrated by siRNA knock-down of Salp15 in ticks, resulting in less transmission of the pathogen to experimental animals. In order to determine whether Japanin was relevant for pathogen transmission, the protein could be knocked-down in *R. appendiculatus* ticks for subsequent challenge of animals with a *R. appendiculatus*-transmitted pathogen, such as *T. parva*. Moreover, it could be assessed whether Japanin bound directly to pathogens and thereby prevented them directly from elimination by the host.

#### 4.1.3 Possible therapeutic applications of Japanin

Because of their central role in the activation or inhibition of T cells, DC represent a potential target for immunotherapeutic approaches in treating infectious disease, cancer,

transplant rejection, allergies, and autoimmune diseases <sup>319-324</sup>. A targeted induction of peripheral tolerance via DC could be beneficial for numerous diseases associated with inappropriate or undesirable immune responses, as in transplant rejection, allergy, and autoimmunity <sup>325-329</sup>. A number of cytokine-neutralising antibodies that prevent polarisation of Th1 and Th17 have already been approved for the treatment of autoimmune diseases, and more are under development <sup>330-338</sup>. Based on the immunomodulatory functions of Japanin, we see possible alternative approaches for the treatment of autoimmune diseases. As shown in this study and by others, Japanin inhibits not only secretion of cytokines associated with Th17 (IL-1 $\beta$ , IL-6) and Th1 (IL-12p70, IFN $\gamma$ ) polarisation as well as innate pro-inflammatory cytokines (IL-1 $\beta$ , IL-6, TNF- $\alpha$ ), but also decreases the expression of the co-stimulatory surface molecule CD86. At the same time Japanin enhances expression of the co-inhibitory surface molecule CD274 and secretion of the anti-inflammatory cytokine IL-10. Consistent with these findings, we observed within this study that the ability of DC to activate T cells is hindered when Japanin is present in an MLR. Although unpublished data have shown that Japanin does not induce the expression of FoxP3 in T cells in an MLR (personal communication, Oliwia Rysnik), given that Japanin increases the secretion of IL-10 by DC, we hypothesise that Japanin may induce Tr1 cells, which usually do not express FoxP3. The modulation of DC maturation by Japanin is not limited to a certain stimulus, but was observed in response to a wide spectrum of stimuli. This broad range of action may be useful for the development of therapeutic drugs for autoimmune conditions. One example is multiple sclerosis (MS), a neurological autoimmune disease that is mediated by autoreactive Th1 and Th17 cells <sup>339</sup>. Studies with an experimental autoimmune encephalitis (EAE) model have shown that DC are essential to the disease mechanism, as they prime T helper cells with myelin antigens

and direct T cell polarisation into effector subsets <sup>340,341</sup>. However, there are also reports of DC-deficient EAE models which show only mildly attenuated or even worse disease, indicating that other APC in draining lymph nodes may be able to prime encephalitogenic T cells alternatively <sup>342,343</sup>. Yet the subsequent polarisation of T cells might mainly be determined by DC populations in the spleen, as DC depletion also resulted in decreased numbers of regulatory T cells. Apparently this was due to the lack of CD274, which is mainly expressed by DC within the APC population in the spleen, directing immunogenic or tolerogenic responses by reduced or enhanced expression of CD274, respectively. Furthermore, recent studies in a skin delayed-type hypersensitivity (DTH) model showed that CD274 is also essentially involved in the desensitisation of T cells by arresting migration and cytokine production of T cells <sup>344</sup>. Given that Japanin increases the expression of CD274 on DC, we hypothesise that Japanin might regulate T cell responses and thereby may help control disease in DTH and/or in EAE models. Potentially, the induction of IL-10 in DC by Japanin might additionally contribute to this. Moreover, Japanin may attenuate disease further by inhibiting the secretion of Th17- and Th1-polarising cytokines and reducing the expression of CD86 in DC. Collectively, Japanin demonstrates great potential for the treatment of autoimmune diseases and some forms of hypersensitivity; hence it would be highly interesting to test Japanin in experimental disease models.

Preliminary studies of the activity of Japanin in mice are currently being conducted by Patawee Asamaphan in the lab of Dr Daniel Anthony at the Department of Pharmacology, University of Oxford (personal communication, Patawee Asamaphan). Labelled microbeads have been injected into experimental animals either alone or together with Japanin. The effect of Japanin on the distribution of the beads within the mice has then been

monitored in lymph node sections of mice after single injection of beads and co-injection with Japanin. Generally, when micro-beads are subcutaneously injected, they may be taken up by trafficking monocytes, which then either remain in the skin and differentiate into macrophages, or migrate to lymph nodes and differentiate into DC<sup>345</sup>. Upon arrival in the lymph node, about 20-50% of DC may initially reside in the subcapsular sinus (SCS) around high endothelial venules (HEVs) in position to encounter newly arrived T cells, while the others migrate directly into T cell zones in the deep cortex<sup>346,347</sup>. More recent studies suggest that DC around HEVs in lymph nodes may have a crucial role in the regulation of the entry mechanism of naive T cell through HEVs<sup>348</sup>. Given that LPS has been shown to limit differentiation of monocytes into DC, it is not surprising that LPS or whole bacteria have been reported to inhibit the migration of moDC to lymph nodes in a subcutaneous administration model<sup>34,349</sup>. On the contrary, when Japanin was subcutaneously co-injected with beads into footpads of mice, significantly more beads were found in the lymph nodes, compared to a control group of animals that had been administered beads only. This was a surprise since our *in vitro* studies suggested that Japanin is a more potent inhibitor of moDC differentiation than LPS. Interestingly, beads in the Japanin condition located mainly in the SCS, rather than in the paracortical T cell areas, which might suggest that moDC that had taken up beads in the presence of Japanin may be unable to migrate into the deep cortex to activate T cells. The potential Japanin-mediated inability of DC to interact with T cells *in vivo* would be in line with our human MLR studies, which have shown that the presence of Japanin prevents T cell proliferation and polarisation into effector subsets. These very recent *in vivo* results strongly suggest that Japanin has activity in mice, although we did not see any activity of Japanin on mouse bone marrow-derived DC or macrophages in our studies *in vitro*. It will be very

interesting to see whether the preliminary *in vivo* data can be confirmed in future experiments. We would suggest for future studies to include an additional control in this model, such as co-injection of beads with another lipocalin, for example OmCI, used throughout our binding studies as a negative control. Besides the interesting results, Japanin has not caused any harmful side effects in the preliminary *in vivo* experiments. We would expect that Japanin is well tolerated by animals and humans, since ticks have co-evolved with mammalian hosts and generally cause only little harm - apart from the pathogens they transmit. Moreover, our *in vitro* studies have shown that the effects of Japanin are reversible, potentially allowing an unproblematic abortion of the treatment in case of any unwanted side effects.

## 4.2 Identification of CD206 as a Japanin-binding receptor

Using a combination of different techniques, we have been able to show that CD206 is a Japanin-binding receptor on moDC. Seeking to identify receptors that specifically bound Japanin we used OmCI as a negative control in all binding studies because it is also a tick-derived lipocalin yet does not interact with DC. We have confirmed within this study that the modulatory functions of Japanin on T cells are DC-dependent. Therefore, we hypothesised that Japanin-specific receptors were exclusively expressed by DC. Consistent with this hypothesis, we found that Japanin bound specifically to DC and monocyte subsets in human blood, but no other leucocyte population. However, the binding of Japanin to blood DC was significantly lower than the binding to moDC. A screen of monocytic and DC-like cell lines revealed that most of them did not bind Japanin. Only Mutz-3-derived iDC showed substantial binding of Japanin, yet it was still less compared to moDC. Thus, moDC were used in the following pull-down experiments in order to isolate and identify Japanin-specific receptors. Subsequent mass spectrometric analysis

strongly suggested CD206 as a Japanin-binding receptor. This was confirmed by binding experiments with recombinant human and mouse CD206 constructs, which showed that Japanin bound to the carbohydrate-binding CTLD4-7 domains of the receptor. The binding of Japanin to recombinant CD206 constructs, as well as to DC, could be inhibited by EDTA and competed by sugars, suggesting that it was glycan-dependent. Despite the glycan-dependency of the binding, we cannot rule out the possibility that the glycosylation of Japanin is only a consequence of the expression system we used, which means it may not be glycosylated when naturally produced by ticks. On the other hand, our studies suggest that glycosylation is crucial for the proper expression or stability of Japanin, since we have been unable to produce stable preparations of non-glycosylated Japanin. Hence we would suspect that natural Japanin is glycosylated. Furthermore, the binding of Japanin correlated with the expression of CD206 by different cell types and vanished when the receptor was knocked-down in moDC. However, contrary to our initial hypothesis that Japanin binds specifically to DC, CD206 is not exclusively expressed by DC, but also by macrophages and certain epithelial cells. Binding assays with BMDC and BMM confirmed that the binding of Japanin correlated with the expression of CD206. Opposed to moDC and macrophages, BMDC express only low levels of CD206 in steady state. Consistently, we found almost undetectable binding of Japanin to unstimulated BMDC, whilst BMM bound Japanin abundantly. This observation suggested that the modulatory functions of Japanin were not restricted to DC, but might also affect other cells, which express suitable receptors.

### 4.3 The role of CD206 in the activity of Japanin

#### 4.3.1 Potential dispensability of CD206 in the activity of Japanin

Surprisingly after the identification of CD206 as a Japanin-binding receptor, the binding of Japanin did not appear to correlate with the activity of Japanin. Firstly, the expression of CD206 alone did not make mouse BMM susceptible for Japanin, as Japanin could not modulate the expression of CD86 or the secretion of TNF- $\alpha$  by BMM, although these cells expressed CD206 and bound Japanin to a similar level as moDC. Secondly, while diminished binding of Japanin was observed after knock-down of CD206 in moDC, the modulatory functions of Japanin did not decrease in CD206 knock-down moDC, as measured by the expression of CD86 and CD274. Similarly, we found that Japanin modulated the expression of CD14, CD86, and CD274 and the secretion of TNF- $\alpha$  by monocytes and the monocytic cell line THP-1, which do not express CD206 and bind Japanin only to a minimal degree, as also seen in blood DC populations. The low level of binding to blood DC, monocytes and THP-1 cells might indicate that there are Japanin-specific surface receptors other than CD206. Moreover, Japanin appeared to rapidly induce phosphorylation of STAT3 in moDC and monocytes. This promotes the idea that Japanin triggers a specific receptor on or in both cell types, which induces the phosphorylation of STAT3. Since we have not specifically tested whether the activation of STAT3 is required for the modulatory functions of Japanin, we cannot be certain that STAT3 is involved in the mode of action of Japanin. Yet there are studies which suggest that STAT3 negatively regulates DC functions <sup>350</sup>. Furthermore, the efficacy of Japanin appeared to be higher in THP-1 cells than in moDC. This was however most likely not due to the absence of CD206 on THP-1, since the efficacy of Japanin was not significantly different in CD206 knock-down moDC compared to moDC that were treated with mock

siRNA. Collectively, our data suggest that CD206 is dispensable for the modulatory functions of Japanin; at least regarding the expression of CD14, CD86, and CD274, and the secretion of TNF- $\alpha$ , since these are the parameters we tested. This means it might be possible that not all functions of Japanin are mediated through the same receptor. Consequently we cannot exclude that CD206 is involved in the mode of action of Japanin, but this involvement may be limited to specific cytokines or other effects, which we did not investigate. Hence we would suggest screening CD206-negative cells, such as THP-1, for the expression of other surface molecules and cytokines that have been shown to be modulated by Japanin.

#### **4.3.2 Possible role for CD206 in potential functions of Japanin on other cells**

Tick-derived immunomodulators are often multifunctional and bifunctionality has also been demonstrated for some lipocalins. For example, the binding of Salp15 to T cells inhibits their proliferation, production of IL-2, and expression of CD25. While these functions are DC-independent, it was found that Salp15 also binds to DC-SIGN on DC and modulates their cytokine expression, which might additionally indirectly inhibit T cells. Moreover, Salp15 associates with a tick-borne bacterium and protects it from antibody-mediated killing by the host. As for bifunctional lipocalins, one example is L-PGDS, which may act as a prostaglandin synthase or as an RA transporter. OmCI represents both, a bifunctional SGE-derived immunomodulator and a bifunctional lipocalin, as it inhibits complement activation pathways and also neutralises inflammatory mediators in the serum. We therefore hypothesise that also Japanin may have more than one mode of action. For example, the binding of Japanin to CD206 might have modulatory functions in cells other than DC or macrophages. In fact, conventional DC express CD206 only at low

levels. On the other hand, endothelial cells in lymphatic vessels express CD206 in order to facilitate lymphocyte trafficking<sup>351,352</sup>. Compounds in SGE of *Dermacentor andersoni* and *I. scapularis* have been shown to modulate the expression of ICAM-1 as well as VCAM-1 and P-selectin<sup>207,353</sup>. Hence the binding of Japanin to CD206 might have functions on endothelial cells, which we have not tested within this study.

#### 4.3.3 Potential involvement of CD206 in a Japanin delivery mechanism

CD206 is a recycling scavenging receptor, specialised on carbohydrate binding and intracellular sorting functions. Thus, CD206 might scavenge Japanin only due to its glycosylation. However, it appears unlikely that most of the binding of Japanin by moDC is mediated by CD206, but this binding has no functional consequences. Previous fluorescence microscopy work in our lab has demonstrated that moDC internalised Japanin in endosomes (personal communication, Oliwia Rysnik). Since CD206 traffics between the plasma membrane and early endosomes, we aimed to investigate whether endocytosis was required for the activity of Japanin. A selection of endocytosis inhibitors was tested, but did not show any effect on the activity of Japanin. Yet these results do not generally exclude that internalisation of Japanin may be required for its activity. Therefore we would suggest for future studies to compare intracellular locations of Japanin in CD206-negative cells, such as THP-1, and CD206-positive cells, such as moDC. This could be achieved by monitoring the uptake of Japanin, for example via fluorescence microscopy, by THP-1 cells and moDC over a time course under physiological conditions. If Japanin were to be found in the same compartments, it might suggest that these locations may be crucial for the activity of Japanin. Consequently, the actual activity of Japanin might then be conferred by an internal binding partner in this location. Although

the absence of CD206 on moDC did not hinder the activity of Japanin, it might still be involved in delivering Japanin to this receptor when present, but its role may also be taken by other sorting receptors when absent.

#### 4.4 Potential CD206-independent mechanisms of action of Japanin

##### 4.4.1 Exclusion of IL-10 and IL-10R from the mode of action of Japanin

Recent studies suggest that regulatory T cells primarily use IL-10 in order to inhibit priming of effector T cells by DC <sup>354,355</sup>. Moreover, many pathogens produce molecules with homology to human IL-10, such as virus-encoded IL-10, or pathogens may directly induce IL-10 in host cells in order to avoid elimination <sup>103</sup>. Also LPS induces IL-10 in DC, after initially activating DC and promoting the expression of CD86 as well as inflammatory cytokines. This is possibly mediated by a negative feedback mechanism in DC in order to prevent excessive immune responses <sup>355</sup>. From a functional point of view, Japanin appears to share many aspects with IL-10 and it also increases IL-10 secretion by DC. Structurally, both molecules are of similar size and dimeric. Based on these similarities, we hypothesised that IL-10 or the IL-10 receptor might be crucial for the mode of action of Japanin and speculated that Japanin might mimic IL-10. Alternatively, the primary function of Japanin might be the enhancement of IL-10 secretion and the other functions, such as the decrease in CD86 expression, were downstream effects of IL-10. In this context, we moreover hypothesised that Japanin might block the IL-10 receptor by binding to it, since IL-10 regulates its own expression via feedback through the IL-10 receptor <sup>356</sup>. Blocking the IL-10 receptor may result in prolonged IL-10 expression, since it has been shown that this disables OspA- or LPS-stimulated THP-1 cells to downregulate the production of IL-10 <sup>357</sup>. In order to test our hypotheses, we measured the influence of

recombinant IL-10 and Japanin on the expression of CD14, CD86, and CD274 in the presence of anti-IL-10 or anti-IL-10 receptor antibodies. While these antibodies did inhibit the functions of recombinant IL-10, they did not inhibit the activities of Japanin. This suggests that the regulation of surface molecule expression on DC through Japanin is IL-10 or IL-10 receptor-independent. Potentially this might provide novel and alternative approaches for the treatment of autoimmune diseases that result from genetic IL-10 or IL-10 receptor defects, for example some forms of inflammatory bowel disease, rheumatoid arthritis, or autoimmune encephalomyelitis <sup>358,359</sup>.

#### 4.4.2 Potential intracellular catalytic activity of Japanin

Lipocalins may act as enzymes and synthesise prostaglandins, which can have immunosuppressive properties <sup>144,254,256</sup>. We hypothesised that Japanin might function as an enzyme and may synthesise an immunoactive compound, for example by catalysing a substrate in the medium. In this context, we speculated that the contact with cells might be dispensable for the mode of action of Japanin. Therefore we tested different Japanin-conditioned media, which had been cleared from Japanin, for activity on moDC. However, no modulatory activity on the expression of CD86 by moDC could be observed under these conditions, suggesting that the mode of action of Japanin requires direct cell contact, but not disproving that Japanin may have catalytic capacities. On the contrary, Japanin may exert catalytic activities on intracellular molecules that regulate the differentiation and maturation of monocytes and moDC.

#### 4.5 Conclusions

This work has contributed to demonstrate that Japanin arrests DC differentiation from monocytes and modulates DC maturation in response to a variety of stimuli. Moreover, we

have demonstrated for the first time that Japanin reduces the ability of DC to prime or activate CD4<sup>+</sup> and CD8<sup>+</sup> T cells in an MLR, while it has no effects on T cells in the absence of DC. Collectively the immunomodulatory activity of Japanin appears to reprogramme DC maturation in response to inflammatory stimuli from immunogenic towards tolerogenic, as if DC would mature under homeostatic conditions. Whilst this immunomodulatory function of Japanin may have originally evolved as part of a strategy of metastriate ticks to evade elimination by the host during feeding, we have elucidated relevance for understanding this mechanism as it might facilitate pathogen transmission by ticks. Hence, targeting Japanin with specific vaccines might potentially prevent tick-borne diseases. Additionally, we have shown potential for therapeutic applications of Japanin, for example in the treatment of autoimmune diseases, as it may control unwanted immune responses that are mediated by DC. Thus we suggest that further studies should focus on the in vivo activities of Japanin, especially in autoimmune models.

A key finding of our work was the identification of CD206 as a Japanin-binding receptor, which we verified by a number of different methods. However, CD206 appeared to be dispensable for the modulatory activity of Japanin. As a possible explanation for this phenomenon, we proposed that not all modulatory functions of Japanin may be mediated by the same receptor, meaning that the binding to CD206 could result in modulation of only certain cytokines or surface molecules, for which we had not screened. Alternatively, CD206 might possibly be involved in potential activities of Japanin on cells other than monocytes, DC, or macrophages, which we had not investigated within our studies. Moreover, we did not exclude the possibility that CD206 may be involved in intracellular delivery of Japanin to another receptor, which might mediate the modulatory activity of

Japanin, since this sorting function might be performed by other receptors in the absence of CD206.

Further endeavour to resolve the mechanism that mediated the activity of Japanin revealed that an essential involvement of IL-10 or the IL-10 receptor could be excluded, as we had hypothesised based on similar functions. Due to the lack of a clear sign for Japanin-specific receptors other than CD206 on the moDC surface, our final experiment addressed the question whether direct cell contact was needed for the mode of action of Japanin, and we found that it was. Therefore, although the binding to the moDC surface appeared to be dispensable for the activity of Japanin, we conclude that internalisation inside the cell may be required for the mechanism of action of Japanin. Given that lipocalins may act as enzymes, we moreover hypothesise that Japanin might catalyse the intracellular synthesis of compounds that regulate DC maturation. Hence we suggest that future investigations of Japanin should focus on pathways of intracellular receptors and accessory regulatory molecules, as these studies might elucidate more crucial factors in the mode of action of Japanin. Hypothetically, this might also enlighten our general understanding of what determines the outcome of DC maturation; whether it is immunogenic or tolerogenic.

#### **4.6 Perspectives for future studies**

Just as the mode of action of Japanin was only partly resolved within this study, it is still not fully understood what exactly decides whether DC mature in an immunogenic or in a tolerogenic manner. One strategy of some pathogens to evade immunogenic responses by DC appears to be the modulation of signalling pathways of specific TLRs by binding to a CLR at the same time. For example, the simultaneous stimulation of TLR4 and DC-SIGN

suppressed the secretion of inflammatory cytokines and promoted the production of IL-10. Japanin on the other hand acts in a much broader fashion and appears to reprogramme DC to undergo tolerogenic maturation almost regardless of additional stimuli. This might indicate that Japanin ‘flips a switch’ in DC that determines their maturation in a tolerogenic fashion. This study suggests that such a receptor may be located intracellularly. But of what sort of nature might this intracellular binding partner of Japanin in DC be?

Recent studies suggest that the fate of DC maturation is determined by TLR downstream-signalling or transcriptional events. One study implies a role for NF- $\kappa$ B1, a precursor of the p50 subunit of NF- $\kappa$ B, in negative regulation of TNF- $\alpha$  expression and activation of effector T cells by steady-state DC <sup>360</sup>. Earlier studies have already associated excessive NF- $\kappa$ B activity with autoimmune disease and showed that homodimers of the p50 subunit of NF- $\kappa$ B can repress transcription of pro-inflammatory genes. Another study has demonstrated that also IRFs can be suppressed by p50 homodimers <sup>361</sup>. Other recent reports have promoted the idea that A20, another NF- $\kappa$ B suppressor, might be crucially involved in the regulation of immune homeostasis and prevention of autoimmune disease <sup>362,363</sup>. Mice lacking A20 specifically in DC showed limited MyD88-dependent signals, resulting in increased production of inflammatory cytokines, as well as limited MyD88-independent signals, leading to increased expression of co-stimulatory molecule CD86 <sup>362</sup>. Furthermore, mice with A20-deficient DC developed symptoms of systemic lupus erythematosus <sup>363</sup>. Another study in mice has reported that deletion of the Tnfr1 gene in DC results in exaggerated NF- $\kappa$ B and MAPK signalling and increased production of IL-23 in a MyD88-dependent fashion, overall promoting susceptibility to psoriasis <sup>364</sup>. Tnfr1 encodes the A20 binding and inhibitor of NF- $\kappa$ B1 (ABIN-1) protein that regulates several

NF- $\kappa$ B signalling events. These studies strongly suggest that NF- $\kappa$ B inhibitors are involved in the induction of a tolerogenic DC phenotype by suppressing the production of inflammatory cytokines. Recent genome-wide analysis of gene expression signatures from TLR-stimulated DC in humans and mice came to the conclusion that convergent transcriptional reprogramming occurs during maturation. It has been proposed that immunogenic DC maturation is generally driven in a conserved manner regardless of the DC subset, stimuli, or mammalian species, involving the modulation of a core set of genes under the control of NF- $\kappa$ B and specific IRFs <sup>365</sup>. Interestingly, our results from Japanin activity assays with human moDC and mouse BMDC suggest that the induction of tolerogenic maturation is not fully conserved either between different DC subsets or between human and mouse, since only human moDC were modulated by Japanin, but not mouse BMDC. More recent comparative studies of immunogenic and tolerogenic DC of different subsets in mice found large overlaps in the expression of core genes between TLR-induced and homeostatic maturation <sup>10</sup>. An interesting difference was however, that the gene which encodes A20 was upregulated in TLR-stimulated DC, but was down-regulated in migratory homeostatic DC. This appears contradictory to the suggested function of A20 in immune homeostasis. However, the upregulation of inhibitory genes during immunogenic maturation might indicate negative feedback mechanisms that regulate the activity of DC upon TLR-stimulation, in order to prevent overwhelming immune responses <sup>10</sup>. The example of A20 demonstrates the entangled complexity of the mechanisms that determine immunogenic or tolerogenic maturation in DC. In addition to gene regulating factors, also post-translational modulations as well as metabolic reprogramming in glycolysis and Krebs cycle have recently been shown play a role in the process of DC maturation <sup>366-369</sup>.

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We could imagine that the mode of action of Japanin involves one or more of the regulatory molecules that have been identified in the pathways associated with DC maturation. Hence, it would be very interesting to investigate how Japanin might potentially interact with these factors in order to reprogramme DC from immunogenic towards tolerogenic maturation.

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