IMMUNOREGULATORY ROLES OF CD73 IN LYMPHATIC ENDOTHELIAL AND MYELOID CELLS

Dominik Eichin

UNIVERSITY OF TURKU

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Dominik Eichin

University of Turku

Faculty of Medicine
Institute of Biomedicine
Medical Microbiology and Immunology
Turku Doctoral Programme of Molecular Medicine
MediCity Research Laboratory

Supervised by

Academician, Sirpa Jalkanen
MediCity Research Laboratory
Institute of Biomedicine
University of Turku
Turku, Finland

Professor, Marko Salmi
MediCity Research Laboratory
Institute of Biomedicine
University of Turku
Turku, Finland

Reviewed by

Associate Professor, Susanna Fagerholm
Department of Biosciences
Faculty of Biological and
Environmental Sciences
University of Helsinki
Helsinki, Finland

Docent, Tuure Kinnunen
Institute of Clinical Medicine
Clinical Microbiology
University of Eastern Finland
Kuopio, Finland

Opponent

Associate Professor, Pipsa Saharinen
Translational Cancer Biology Program
Faculty of Medicine
University of Helsinki
Helsinki, Finland

Für mini eldre, Doris un Bernhard

Es isch jo scho es zittle her,

wo in de nachd me ghört miis gblär.

Doch schnäll ischs dann viil bessa gsi,

wenn d eldre cho sin ane hi.

Un mid viil sorgfald, liäb, gduld,

des büeble hän bald iine glullt.

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doch bi i au hüd no richdig froh,

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un dankbar bi für alles wo,

ma zämme hän, un no düed cho.

Dominik

UNIVERSITY OF TURKU
Faculty of Medicine
Institute of Biomedicine
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ABSTRACT

The immune system is a fascinating product of the interaction and cooperation of a multitude of cells and tissues. It is thereby able to distinguish between "friend" and "foe" and protect the host from a great variety of pathogens. To do so, the activity of the immune system is finely tuned by various cytokines, chemokines and other molecules. One such regulatory molecule is CD73. This ectonucleotidase has been found on different immunologically important cells and tissues, where it acts as a signaling, adhesion and immune-modulating molecule. However, its expression, importance or functions have only been investigated on some parts of the immune system.

This study was undertaken to determine the role of CD73 in different macrophage populations as well as in the lymphatic endothelium. In particular, we wanted to decipher if CD73 is important for the function of differently polarized macrophages and investigate if its expression correlates to their polarization status. In addition, we studied if macrophage-polarization was dependent on CD73. Furthermore, the modulation of endothelial CD73, its function and the potential impact that clinical anti-CD73 antibodies could have on the blood and lymphatic endothelium were studied.

Our results show that CD73 expression differs between differently polarized macrophages, but that its expression is dispensable for their polarization. Furthermore, we could demonstrate that the expression of CD73 on afferent lymphatic endothelium contributes to immune-modulating effects and its absence results in a more pro-inflammatory phenotype of both the endothelial cells as well as other immune cells that interact with them. As this effect was mainly visible when genetic modulations of CD73 were used and only marginal with CD73-targeting antibodies, there is likely only minor concern to use such antibodies in a clinical setting.

The outcome of this study therefore shows, that although CD73 has mainly anti-inflammatory properties, its importance and specific role varies between different cell-types and tissues and therefore has to be carefully evaluated.

KEYWORDS: CD73, NT5E, Macrophages, Monocytes, Dendritic Cells, Endothelial cells, Lymphatics, Immune regulation

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TIIVISTELMÄ

Immuunijärjestelmä on useiden eri solujen ja kudosten yhteistyössä toimiva puolustusjärjestelmä. Se pystyy tunnistamaan ystävät vihollisista ja suojelemaan isäntää monilta eri taudinaiheuttajilta. Monet eri sytokiinit, kemokiinit sekä muut molekyylit säätelevät tarkasti immuunijärjestelmän aktiivisuutta sen puolustautuessa taudinaiheuttajia vastaan. CD73, joka on toiminnallisesti ektonukleotidaasi, on yksi säätelevistä molekyyleistä, jota ilmennetään eri immunologisissa soluissa sekä kudoksissa, joissa se osallistuu solujen signalointiin, niiden tarttumiseen sekä immuunijärjestelmän aktiivisuuden säätelyyn. CD73:n ilmentymistä sekä sen merkitystä on tutkittu ainoastaan osassa immuunijärjestelmää.

Tässä työssä selvitettiin CD73:n merkitystä makrofaageissa sekä lymfaattisissa endoteelisoluissa. Halusimme erityisesti selvittää CD73:n merkitystä polarisoituneissa makrofaagipopulaatioissa sekä korreloiko sen ilmeneminen polarisaation kanssa. Lisäksi tutkimme, vaatiiko makrofaagien polarisaatio CD73:n toimintaa. Tutkimme myös endoteelisolussa olevan CD73:n säätelyä ja merkitystä, sekä CD73:n tunnistavien kliinisesti käytössä olevien vasta-aineiden vaikutuksia veri- ja imusuonien endoteelisoluihin.

Tuloksemme osoittavat, että CD73 ilmennetään eri tavoin polarisoituneissa makrofaagipopulaatioissa, mutta sen ilmeneminen ei ole edellytys polarisaatiolle. Pystyimme myös osoittamaan, että tuovien imusuonten lymfaattisissa endoteelisoluissa oleva CD73 osallistuu immuunipuolustuksen aktiivisuuden säätelyyn ja aiheuttaa tulehdusta edistävää ilmiasua niin endoteelisoluissa kuin muissa niiden kanssa vuorovaikutuksessa olevissa soluissa. Nämä vaikutukset johtuivat pääosin CD73:n geneettisistä muutoksista eivätkä juurikaan CD73:a tunnistavista vasta-aineista, jolloin tämän kaltaisten haittavaikutusten osuus vasta-aineiden kliinisessä käytössä jää todennäköisesti pieneksi.

Tämä tutkimus osoittaa, että vaikka CD73:lla on tulehdusta ylläpitävä vaikutus, sen merkitys sekä varsinaiset tehtävät ovat solu- ja kudosriippuvaisia, ja sen varsinainen osuus immuunijärjestelmän säätelijänä tulee määritellä tarkkaan.

AVAINSANAT: CD73, NT5E, makrofaagi, monosyytti, dendriittisolu, endoteelisolu, imusuonisto, immuunipuolustuksen säätely

ZUSAMMENFASSUNG

Das Immunsystem ist das faszinierende Resultat der erfolgreichen Zusammenarbeit einer Vielzahl von Zellen und Geweben. Es ist dabei in der Lage, zwischen „Freund" und „Feind" zu unterscheiden und kann dadurch den Organismus vor zahllosen Pathogenen beschützen. Um dies zu bewerkstelligen wird die Aktivität des Immunsystems durch Zytokine, Chemokine sowie diverse andere Moleküle genaustens reguliert. Ein derartiges Molekül ist CD73. Diese Ektonukelotidase erfüllt verschiedene Aufgaben in der Signalverarbeitung, Adhäsion sowie der Modulation des Immunsystems. Obwohl es auf verschiedenen immunologisch bedeutsamen Zellen und Geweben nachgewiesen werden konnte, ist seine Funktion nur in einigen Bereichen des Immunsystems bekannt.

Um dieses Wissen zu vergrössern wurde daher diese Doktorarbeit durchgeführt. Dabei wurde die Bedeutung von CD73 für verschiedene Makrophagen sowie für lymphatische Endothelzellen untersucht. Im Speziellen wurde dabei untersucht, ob die Expression von CD73 von der Polarisierung der Makrophagen abhängt und ob deren Funktion sowie Polarisierung dadurch beeinflusst wird. Des Weiteren wurde die Modulierung von CD73 auf Endothelzellen sowie seine dortige Funktion untersucht. Dies wurde insbesonders mit Blick auf die therapeutische Anwendung von anti-CD73 Antikörpern und deren Auswirkung auf das Lymph- sowie das Blutendothel durchgeführt.

Die vorliegenden Ergebnisse unserer Studien konnten dabei zeigen, dass sich die Expression von CD73 je nach Polarisierungsstatus der Makrophagen unterscheidet, diese Expression aber die Polarisierung der Zellen nicht beeinflusst. Ausserdem konnten wir nachweisen, dass CD73 auf Endothelzellen immunregulatorische Funktionen erfüllt und dass seine Abwesenheit zu einem pro-inflammatorischen Phänotyp von Endothelzellen sowie von anderen Immunzellen führt. Dieser Effekt konnte hauptsächlich durch genetische Interventionen und nicht durch die Anwendung von CD73-spezifischen Antikörpern hervorgerufen werden, was schlussfolgern lässt dass eine klinische Anwendung solcher Antikörper vorraussichtlich relativ ungefährlich sein dürfte.

Die Ergebnisse dieser Studien verdeutlichen daher, dass obwohl CD73 primär anti-inflammatorische Funktionen erfüllt, seine Bedeutung sich je nach Zell- und Gewebstyp unterscheidet und daher gewissenhaft evaluiert werden muss.

STICHWÖRTER: CD73, NT5E, Makrophagen, Monozyten, Dendritische Zellen, Endothelzellen, Lymphatische Gefäße, Immunregulation

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Abbreviations

ACDC	Arterial calcification due to deficiency of CD73
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ARDS	Acute respiratory distress syndrome
ATP	Adenosine triphosphate
BEC	Blood endothelial cell
cDC	Classical dendritic cell
CLP	Common lymphoid progenitor cells
DC	Dendritic cell
EAE	Experimental autoimmune encephalomyelitis
EC	Endothelial cell
EMP	Erythro-myeloid-progenitors
GPI	Glycosylphosphatidylinositol
HDLEC	Human dermal lymphatic endothelial cell
HDMEC	Human dermal microvascular endothelial cells
HIF	Hypoxia inducible factor
HSC	Hematopoietic stem cell
IRF	Interferon regulatory factor
KO	Knockout
LEC	Lymphatic endothelial cell
LPM	Large peritoneal macrophages
LTi	Lymphoid tissue inducer cells
M1	Type 1 macrophages (pro-inflammatory)
M2	Type 2 macrophages (anti-inflammatory)
MAC	Membrane attack complex
MBL	Mannose binding lectin
MHC	Major histocompatibility complex
moDC	Monocyte-derived dendritic cell
NCR	Natural cytotoxicity receptor
NETs	Neutrophil extracellular traps
NK	Natural killer cell

pDC	Plasmacytoid dendritic cell
PHA	Phytohemaglutinin
SPM	Small peritoneal macrophages
TT	Tetanus toxoid
WT	Wildtype

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I   **Eichin D**, Laurila J, Jalkanen S, Salmi M. CD73 activity is dispensable for the polarization of M2 macrophages. *PLoS ONE*, 2015; 10(8): e0134721. doi:10.1271/journal.pone.0134721.

II   **Eichin D**, Pessia A, Takeda A, Laakkonen J, Bellmann L, Kankainen M, Imhof B, Stoitzner P, Tang J, Salmi M, Jalkanen S. CD73 contributes to anti-inflammatory properties of afferent lymphatic endothelial cells in humans and mice. *European Journal of Immunology, 2020.* doi:10.1002/eji.201948432.

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1   Introduction

Much like insurance plans are covering us during mishaps in our daily life, the human immune system has our back when it comes to our health. It is an intricate array of components and processes that protects us from a wide range of pathogens and malignant cells throughout our lives. It consists of a multitude of different cells, tissues and organs. Underneath this first layer of complexity lie several more, encompassing a wide variety of receptors, chemokines, cytokines and other molecules that constantly perform time- and dose-dependent interactions. In order to try to understand this life-saving machinery, researchers have studied it for decades. Already in 430 BC in ancient Greece, Thucydides observed immunological functions by noticing that survivors of the plague did not fall ill a second time following their recovery. However, it took until the 19th century to get a better understanding of these processes, when Louis Pasteur and Robert Koch founded the principles of vaccination and infectious diseases. These cornerstones of immunology led to the discovery of countless additional principles and finally to modern immunology.

According to current knowledge, the immune system can be divided into two arms, the innate and the adaptive arm. While the innate immune system functions in a broad and non-antigen specific way and is quick to react, the adaptive immune system is antigen-specific and requires a certain time to react. However, the big benefit of the adaptive immune system is that it forms an immunological memory that reduces or even prevents reoccurring infections. Nevertheless, this separation is not always unambiguous as both systems can share certain features and have been shown to interact closely with each other. While some cell types exhibit properties from both arms of the immune system such as innate natural killer cells (NKs) that have the ability to form memory-like cells, others have been found to closely interact with both systems. Two examples of such interacting cells are macrophages and dendritic cells (DCs) that both belong to the innate cells, but collaborate with cells from the adaptive system. They belong to the group of phagocytes as they can engulf pathogens and foreign particles. Additionally, in particular DCs can migrate via the lymphatic system to draining lymph nodes and present parts of the engulfed object to other cells of the immune system to initiate

an adaptive immune response. As cells residing at the border between the two arms of the immune system, their activity is crucial for the outcome of an infection. It is therefore not surprising, that they exist in a multitude of different phenotypes and their activity is tightly regulated. Hence, their phenotype can range from a strongly inflammation promoting phenotype to an anti-inflammatory and even deactivating phenotype. This versatility makes them a promising target for potential clinical interventions to steer the immune response and therefore disease progression.

An interesting molecule in this aspect is CD73. CD73 exerts immune-regulating roles either via its enzymatic activity or via enzymatically independent functions. Enzymatically, the ectonucleotidase CD73 dephosphorylates extracellular adenosine monophosphate (AMP) to adenosine. The produced adenosine is anti-inflammatory, making CD73 an immunologically highly relevant molecule. As CD73 can be expressed on phagocytes as well as on other immunologically relevant cell types, it has been shown to be involved in immune reactions, cell migration as well as tissue homeostasis. Although it is known that CD73 can be quite abundantly expressed on a multitude of cells and tissues, much still remains unknown. In the work for this thesis I therefore, for the first time, investigated the expression pattern and function of CD73 on macrophages and their polarization. Furthermore, I also shed light on the role of CD73 on afferent lymphatic vessels and the importance it has for cells migrating there.

2 Review of the Literature

2.1 The immune system

The immune system is a very important part of vertebrates and invertebrates alike. It does not only protect the organism from harmful entities such as bacteria and viruses, but also plays an important role in the removal of mutated cells. While the system itself is very old, only during the last centuries have we gained the necessary understanding to truly appreciate it.

Although it has become more complex with every new discovery, the immune system can be crudely divided into an innate and an adaptive part that consist of different types of cells and fulfill specific roles.

2.1.1 The innate immune system

The innate immune system can be considered the first line of defense against the perpetual onslaught of foreign microorganisms and molecules that we are exposed to every single day. This system thereby does not only implement sophisticated ways to deal with a potential threat, but also relies on some rather "basic" solutions (Kenneth et al., 2012). Depending on how a pathogen comes in contact with its host, one of the first lines of defense is the skin. The skin is both a physical as well as a chemical barrier as it, in addition to mechanically preventing the entering of a pathogen, is also covered with an acidic coat and is colonized by a multitude of commensal bacteria. Furthermore, it expresses a plethora of anti-microbial peptides that can exert direct antimicrobial responses or function in a more indirect way by activating a host response (Schauber and Gallo, 2008). Similarly, other organs of the human body have comparable properties and implement various chemical agents as a defense mechanism. For instance, in case a foreign organism enters the eye cavity it will have to deal with being flushed out due to the tear film as well as its lysing properties caused by the presence of lysozyme.

Another likely point of entry via the mouth and nasal cavities is also protected by lysozyme (mouth), the presence of clumping mucus or respiratory epithelium (nose and respiratory tract) or even strong acids, proteases and physical activity (stomach and digestive tract).

Once a pathogen manages to penetrate these barriers, the cellular innate immune system as well as the complement system come into play (Kenneth et al., 2012). The cellular system consists of different types of cells that are either able to phagocytose an invading pathogen, produce immune-stimulating cytokines or kill the pathogen directly. The cells that are hereby involved are different types of granulocytes, innate lymphoid cells, monocytes and macrophages, γδ T cells as well as dendritic cells. All of these cells have in common, that they can react to a wide range of infectious agents, as they are able to recognize evolutionary conserved molecules that are expressed on the surface of the pathogen or secreted cytokines acting as danger-signals. This allows their reaction to be immediate, as they do not require an initial priming step. However, the drawback is that even though they can recognize a wide range of potentially dangerous microbes, they are not very specific in their reaction and therefore certain pathogens are able to evade them. Most of these cell types have been known for quite some time, however, due to advances in this area, in addition to the discovery of the completely new category of innate lymphoid cells, existing categories had to be refined and expanded. Similar to the cellular innate immune system, also the complement system consists of many different components. Discovered in 1800 and named in the 1900s, the complement system consists of around 50 different proteins and protein fragments and has a major role in the defense against infections (Pillemer, 1943). It can be activated by the 'classical pathway', the 'lectin pathway' and the 'alternative pathway' (Bordron et al., 2020; Carroll and Sim, 2011; Molina, 2004). Although these pathways differ in the mediators that trigger them and follow somewhat different steps, all of them have a common outcome, namely to opsonize pathogens, to trigger inflammation by attracting immune cells and to form a membrane attack complex (MAC) that destroys pathogens by generating holes in their cell membranes.

2.1.1.1 Granulocytes

Granulocytes make up the largest portion of leukocytes circulating in the human blood and can be divided into basophils, eosinophils, neutrophils and tissue-resident mast cells (Kenneth et al., 2012; Lin and Loré, 2017). Basophils and eosinophils are rather rare cell populations, making up only a few percent of all the circulating leukocytes. While the main function of basophils is to promote an inflammatory response by the release of heparin, histamine or serotonin, eosinophils are able to phagocytose and secrete cytotoxic and lysing substances such as cathepsin. The third type of granulocytes are neutrophils, which can make up to 70% of the circulating leukocytes. These cells are very versatile, as they are capable phagocytes, can release anti-microbial substances and additionally can

generate so called neutrophil extracellular traps (NETs) (Papayannopoulos, 2018). NETs are mainly made out of DNA and enable the neutrophil to immobilize and destroy extracellular microbes without the need for phagocytosis. The last type of granulocytes are mast cells. Mast cells are tissue-resident cells that can be found primarily in areas that come in contact with the environment, such as the skin or the lungs. Mast cells are similar to basophils and are capable of releasing large amounts of mediators such as cytokines, growth factors and proteases and are thereby often an important player in allergies. Although mast cells are capable of phagocytosis and play a role in bacterial and viral clearance, arguably their main task is the recruitment of other innate immune cells (Cardamone et al., 2016).

2.1.1.2 Innate lymphoid cells

Innate lymphoid cells are a relatively recently discovered group of cells that can be considered innate immunity analogues of T cells. Although they do not possess diverse antigen-recognition receptors like T cells, they are important for different immune functions. Until 2008, natural killer cells (NKs) and lymphoid tissue inducer cells (LTis) had been the only cells characterized originating from common lymphoid progenitor cells (CLPs) that did not express any antigen receptors. This changed, as three more similar cell types were discovered (Bernink et al., 2013; Mazzurana et al., 2018). These cells, also originating from CLPs, were found to express NK- and LTi-markers, resembled T-helper cells or were found to be similar to NK cells but lacked their cytotoxic capabilities. In the new nomenclature, all these cells were categorized into three groups, ILC1s, ILC2s and ILC3s. This classification was chosen to resemble the T-helper subsets Th1, Th2 and Th17 and is based on their cytokine expression and transcription factor profiles. Although not all of these groups are very well defined at this moment, the current definition of ILC1s places NK cells and the IFN-γ and TNF-α producing intraepithelial CD127^{low} and CD127^{high} cells that are mainly found in the lamina propria into this group (Mazzurana et al., 2018). The Th2-resembling group of ILC2s can be predominantly found in areas of the skin, the lungs, the liver and the gut and secrete cytokines such as IL-4, IL-5, IL-9 and IL-13 (Mazzurana et al., 2018). Finally, the group of ILC3s comprises of LTi and ILC3 cells. As the name suggests, LTis play an important role in the development of secondary lymphoid tissue, however, their role in adults is currently not clear (Lane et al., 2009; Withers, 2011). Although it is known that they promote the survival of CD4$^+$ memory T cells, they are likely not limited to this function. The last cell type, ILC3s, can mainly be found in the intestine and in mucosal tissues. The expression of the natural cytotoxicity receptor (NCR) allows the discrimination of two subgroups, namely NCR-positive and NCR-negative ILC3 cells. ILC3s produce

IL-22 and IL-17 upon stimulation and it has been shown that if given the right stimuli they are capable of converting into ILC1 cells (Bernink et al., 2013; Eberl et al., 2015; Hazenberg and Spits, 2014; Lane et al., 2009; Mazzurana et al., 2018; Neill and Fallon, 2018; Spits and Cupedo, 2012; Withers, 2011).

2.1.1.3   Monocytes

Monocytes belong to the group of leukocytes and derive from hematopoietic stem cells (HSC) in the bone marrow. They can be found circulating in the blood and under the right circumstances can infiltrate tissues and differentiate into macrophages or dendritic cells. To date, in humans three different subpopulations have been identified according to their expression of CD14 and CD16: $CD14^{++}CD16^{-}$ classical monocytes, $CD14^{+}CD16^{++}$ non-classical monocytes and $CD14^{++}CD16^{+}$ intermediate monocytes and these subsets secrete distinctive amounts of TNF-α, IL-6 and IL-1β after stimulation. In mice, similar subtypes have been identified according to their expression of Ly6C, CD43, CD62L and CCR2 and grouped into $Ly6C^{+}CD62L^{+}CD43^{-}CCR2^{+}$ classical, $Ly6C^{int}CD62L^{-}CD43^{+}CCR2^{-}$ intermediate and $Ly6C^{-}CD62L^{-}CD43^{+}CCR2^{-}$ non-classical monocytes (Ginhoux and Jung, 2014; Hettinger et al., 2013; Kawamura et al., 2017; Misharin et al., 2014).

All of the subsets fulfill the same basic functions of phagocytosis, cytokine production and antigen presentation, but differ in their differentiation potential. While all can become macrophages, only the classical monocyte can transform into a monocyte-derived dendritic cell (moDC).

2.1.1.4   Macrophages

Macrophages are a very diverse population of cells within the category of leukocytes that possess phagocytic capabilities. Discovered by Metchnikoff, these cells can be found in almost all tissues including the brain (microglia), the bone (osteoclasts) and the liver (Kupffer cells). While one of their main characteristics is their phagocytic ability (hence the name), they are involved in processes of the innate as well as the adaptive immune system by being able to attract and activate other immune cells. They can do so via the secretion of certain cytokines or by presenting processed antigen. In addition to these mainly inflammation-associated functions, macrophages can also take part in tissue homeostasis and repair.

Macrophages can originate from three different origins: they can differentiate from bone marrow-derived blood-circulating monocytes, they can derive form fetal liver monocytes or they can be yolk-sac derived. Interestingly, most macrophages that can be found in steady-state conditions in the tissue descend from erythro-

myeloid-progenitors (EMP) and are therefore yolk sac or fetal liver derived. These cells are already present in tissues before birth (Ginhoux and Guilliams, 2016).

Under normal conditions, tissue-resident macrophages are self-renewing but under inflammatory conditions and the associated influx of monocytes and monocyte-derived macrophages, a replenishment of these cells by incoming cells is possible. Interestingly, while it originally has been thought that after the inflammatory event has passed, the body returns to its steady state and the original population of tissue macrophages will be reinstated, it has now been shown that although most monocyte-derived macrophages are rather short-lived, some of these cells possess the ability for self-renewal (Bain et al., 2016; Ginhoux et al., 2015; Hoeffel and Ginhoux, 2018; van de Laar et al., 2016; Röszer, 2018).

Macrophages are a very versatile cell-type and cannot only originate from different sources, but one of their most interesting (and complex) feature is their adaptability. The two extremes of this adaptability are the pro-inflammatory phenotype, often referred to as M1, and the anti-inflammatory phenotype, referred to as M2 (Figure 1). Originally this dichotomy was described in mice, where macrophages from C57BL/6J strains reacted to external stimuli by inducing iNOS and a Th1 response, while macrophages from BALB/c mice induced arginase and a Th2 response (Mills et al., 2000). This nomenclature is therefore based on the established classification of CD4$^+$ T cells into Th1 and Th2 cells, as the prototypic cytokines from these cells (IFN-γ/TNF-α for Th1 and IL4/IL13 for Th2) polarize macrophages towards M1 or M2, respectively. M1 cells express molecules such as MHC class II, CD80 and iNOS and can produce vast amounts of inflammatory cytokines such as TNF-α, IL-6 and IL-12. This enables them to neutralize, engulf and digest pathogens and present their processed antigens to other cells of the immune system. They therefore not only form the initial defense by phagocytosing microorganisms but are also activating T cell responses and remove cell debris. M2 cells, on the other hand, are often identified by their expression of CD206 or CD163 and they secrete cytokines such as IL-10, TGF-β or VEGF-A. This makes them anti-inflammatory and allows them to promote tissue repair, wound healing as well as angiogenesis (Murray, 2017). Furthermore, this cell type takes part in the defense against helminths and fungi (Atri et al., 2018). Although the phenotypes of macrophages are very similar between human and mouse, there are some differences regarding the expression of different surface molecules that can be used for their identification. While human macrophages express the phenotypic markers CD64, CXCL10, IDO and SOCS1 on M1 cells and CCL22, CD23, MRC1 and TGM2 on M2 cells, mouse M1 cells express CXCL9, CXCL10, CXCL11 and NOS2, and M2 cells express Arg1, Fizz1, Mrc1 and YM1/2 (Albright et al., 2016; Chávez-Galán et al., 2015; Duluc et al., 2007; Martinez and Gordon, 2014; Martinez et al., 2013, 2014; Rőszer, 2015).
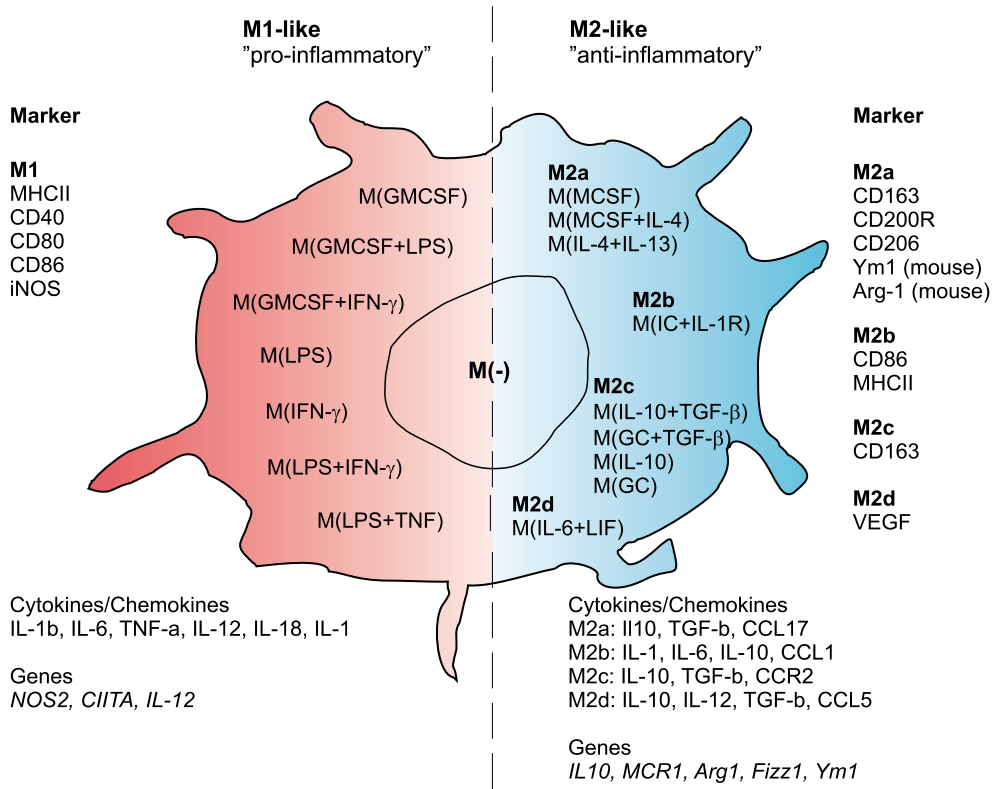
M1-like
"pro-inflammatory"

M2-like
"anti-inflammatory"

Marker

M1
MHCII
CD40
CD80
CD86
iNOS

M(GMCSF)

M(GMCSF+LPS)

M(GMCSF+IFN-γ)

M(LPS)

M(IFN-γ)

M(LPS+IFN-γ)

M(LPS+TNF)

M(-)

M2a
M(MCSF)
M(MCSF+IL-4)
M(IL-4+IL-13)

M2b
M(IC+IL-1R)

M2c
M(IL-10+TGF-β)
M(GC+TGF-β)
M(IL-10)
M(GC)

M2d
M(IL-6+LIF)

Marker

M2a
CD163
CD200R
CD206
Ym1 (mouse)
Arg-1 (mouse)

M2b
CD86
MHCII

M2c
CD163

M2d
VEGF

Cytokines/Chemokines
IL-1b, IL-6, TNF-a, IL-12, IL-18, IL-1

Genes
NOS2, CIITA, IL-12

Cytokines/Chemokines
M2a: Il10, TGF-b, CCL17
M2b: IL-1, IL-6, IL-10, CCL1
M2c: IL-10, TGF-b, CCR2
M2d: IL-10, IL-12, TGF-b, CCL5

Genes
IL10, MCR1, Arg1, Fizz1, Ym1

Figure 1. Macrophages can polarize from their "basic" M(-) status into different distinct and intermediate phenotypes. Examples of typical polarizing agents and the nomenclature of those cells can be seen in the middle. In addition typical markers, cytokines/chemokines and genes are shown. Unless mentioned otherwise, all listed molecules can be found in human and mouse. IC=immune complex, GC=glucocorticoids. Adapted from (Albright et al., 2016, Cháyez-Galán et al., 2015, Duluc et al., 2007 and Röszer 2015).

However, instead of a "black and white" clear-cut system, the reality is much more complicated and blurred. In 2004 Mantovani and others published, that there is not only a single type of M2 macrophage, but rather a multitude of them that they coined M2a, M2b, and M2c (Mantovani et al., 2004). Additionally, also M2d cells have been described by some groups (Martinez et al., 2008). While M2a cells resemble the previously termed M2 cells, M2b cells are induced by the exposure to immune complexes and TLR-ligands. This gives these cells the ability to produce pro- as well as anti-inflammatory cytokines. M2c cells can be induced through the exposure to IL-10 and TGF-β, after which they themselves produce those cytokines and have therefore a strong anti-inflammatory "deactivating" phenotype and limit the presentation of antigens in monocytes and macrophages. They can additionally be identified by their expression of CD163 that is the highest amongst all M2-like

macrophages. The last type of the M2-like macrophages are tumor-associated macrophages, sometimes called M2d macrophages (Martinez et al., 2008), which represent a very diverse population of mixed phenotypes (Kim and Bae, 2016).

To make things even more interesting, it turned out that the polarization status of macrophages is not a fixed and permanent state, but rather a fluid continuum. Once polarized, the fate of macrophages is not terminal but macrophages rather retain their ability to adjust to environmental cues and are able to alter between a pro- and anti-inflammatory, as well as a mixed phenotype (Porcheray et al., 2005; Rőszer, 2015). Once this ability was discovered, it opened up a whole range of possibilities to deal with different disease states. In cancer, where up to around 50% of the cancer tissue can consist of macrophages (Kim and Bae, 2016; Yang and Zhang, 2017), these macrophages are usually of a M2 phenotype. With the discovered flexibility of these cells, new treatment strategies came to life whereby the goal was to either reduce the amount of M2 cells in the cancerous tissue environment or to polarize them to an M1 phenotype, thereby limiting cancer growth or even reversing it (Gazzaniga et al., 2007; Luo et al., 2006; Zeisberger et al., 2006).

However, while this approach seems beneficial with regards to cancer, there are other diseases where promoting a more M1-like phenotype is actually detrimental. In inflammatory diseases, such as inflammatory bowel disease or atherosclerosis, M1 inflammatory macrophages are   important players promoting disease progression (Lee et al., 2018).

In these disease conditions, the aim is therefore opposite to the aim in cancer studies: to switch macrophage polarization towards a M2 phenotype.

Hence, a balanced and well-timed polarization between a M1 and M2 phenotype is essential to the body's ability to deal with diseases while preventing an overshooting of the immune response and extensive tissue damage. In general, following the onset of inflammation, macrophages obtain a M1 phenotype that allows them to produce and release pro-inflammatory mediators, present antigens via their MHC receptors and thereby facilitate the killing of invading germs. In time, this process is then limited by shifting the macrophage phenotype towards M2, causing the release of anti-inflammatory mediators that support wound healing and tissue repair (Atri et al., 2018). However, the polarization phenotype of the macrophages as well as the kinetics of this process strongly depends on available stimuli.

Not surprisingly, humans were not the first to discover this flexibility and try to use it to their advantage. Many pathogens are able to modulate this polarization process, for example by expressing arginase. By producing arginase, the pathogens can limit the production of iNOS, as both enzymes require arginine for their function and therefore compete for it (Das et al., 2010; Iniesta et al., 2001). This therefore can shift the polarization of macrophages from NOS-producing M1 cells

to a more anti-inflammatory M2 phenotype, as the limitation of NOS allows for those cells to repolarize (Van den Bossche et al., 2016; Kelly and O'Neill, 2015). This polarizing property has been observed with many different pathogens that each can induce a rather specific polarization profile. The outcome is therefore dependent on the interaction of the immune system with the pathogen. During acute immune responses it is often a predominant M1 macrophage profile that can be observed, as reported with *Salmonella typhimurium* or *Listeria monocytogenes* (Benoit et al., 2008), HIV-1 (Cassol et al., 2010) or the infection with human cytomegalovirus (Chan et al., 2008). In contrast, during a later phase of an infection the profile becomes more M2-like as has been described with *Mycobacterium tuberculosis* infections (Redente et al., 2010) or late stage human cytomegalovirus infections (Avdic et al., 2013).

In order to study this flexibility and to enable a targeted polarization in a controlled laboratory environment, different experimental methods have been established. As described above, one of the original methods to polarize macrophages uses cytokines. In addition, the use of monoclonal antibodies such as using CD47 to enhance M1 polarization or the targeting of specific genes or pathways (for example through miRNAs) have been investigated (Haegel et al., 2013; Jenkins et al., 2011; Li et al., 2018; Mohanty et al., 2019).

The successful polarization of macrophages can subsequently be verified by determining surface molecule expression, gene expression or by morphological analysis. In recent years, the characterization and identification of different cell subtypes has become easier as new technologies were introduced. One of the most powerful is single cell sequencing. This technique allows the analysis of the mRNA profile on the level of a single cell and therefore is ideal to decipher and detect small differences in macrophage populations and their changes due to environmental cues. It is therefore not only facilitating deciphering macrophage development or the detection of previously unknown populations, but also reveals their polarization profiles in more detail, potentially leading to new ways to influence the macrophage phenotype (Bian et al., 2020). Examples of such new findings are the discoveries of new macrophage subsets in the lung after inflammation and a more complex macrophage activation spectrum in atherosclerosis (Lin et al., 2019; Mould et al., 2019). It is, however, important to note that not every treatment modality that can be implemented in a controlled laboratory environment is practical or even feasible in a clinical setting.

Furthermore, it is important to tightly control and observe these cell polarizations as an overshooting of the polarization into the opposite direction might have the potential to cause different diseases and problems. As an example in the case of cancer, a systemic increase of pro-inflammatory cells might lead to a widespread inflammatory reaction and additionally prevent tissue repair. Similarly,

the shift to an anti-inflammatory direction when treating e.g. inflammatory bowel disease, could cause a systemic suppression of the immune system and make the patient more susceptible to potential infections. Finally, while the polarization status of the macrophages plays an important role in disease progression and its outcome, the origin of the cell is also important. In a study investigating allergic lung inflammation, macrophages that were derived from monocytes were promoting the inflammatory reaction, while self-renewing tissue-resident macrophages were protective (Zasłona et al., 2014).

As a final remark, even though the M1/M2 nomenclature is quite intuitive and therefore allows for a swift overview of the topic, it also has some problems. The main problem lies within its greatest advantage: the oversimplification. This simplification can lead to the distinction of merely two different types of macrophages, M1 and M2, while ignoring the plethora of different subtypes and macrophages origins. To solve this, in 2014 a consortium of renowned scientists working with macrophages published a paper describing their recommendations for the nomenclature and experimental guidelines (Murray et al., 2014). Overall their suggestions were a more detailed description of the experimental procedures used for obtaining and/or generating macrophages, the use of defined reagents and cytokines instead of e.g. culture supernatants and other ill-defined agents for the culture and polarization of these cells, the avoidance of certain terminology as well as the use of multiple markers to define the cells. Most importantly, they also proposed a naming system that would indicate the cytokines that had been used for generating the cells, i.e. instead of M1 cells it could for example be M(LPS) or M(TNF) and instead of M2a it could be M(IL4) or M(IL13). However, although their paper seems to have been well-received and has been cited more than 1600 times, there are still numerous papers published in which the M1/M2 nomenclature is used. This might have multiple reasons, such as that researchers are creatures of habit and just continue using a system they have been using in the past, unawareness or refusal of these new suggestions or simply the fact that, although the new nomenclature is more accurate, it is also more inconvenient.

2.1.1.5   Dendritic cells

The third group of cells belonging to the mononuclear phagocyte system (in addition to monocytes and macrophages) are dendritic cells (DCs). Much like other phagocytic cells, these hematopoietic cells can be divided into multiple subtypes. One of these subtypes was discovered in 1868: Langerhans cells. Named after their discoverer Paul Langerhans, these cells were at first mistaken as neurons due to their shape and only many years later, along with other members of the DC group, were identified as antigen-presenting cells. In 1973, Ralph Steinman was the first

to characterize DCs and their importance for the generation of primary immune responses. While these cells are also able to phagocytose, their main role is not the clearance of foreign particles, viruses or bacteria but rather the processing and presenting of antigens and thereby the activation of other cells of the immune system. To do so, these cells can rely on a multitude of mechanisms such as receptor-mediated phagocytosis (e.g. via the mannose receptor) or macropinocytosis, followed by the presentation of the processed antigen on either MHC class I or MHC class II receptors (Bancherau and Steinman, 1998).

Much like with macrophages, also the classification of dendritic cells is not that simple and has been altered and adjusted over the years. In general, DCs can either be considered as tissue-derived, motile cells and found in peripheral tissues such as the skin, or they can be sessile cells found in lymph nodes or the spleen (Villadangos and Heath, 2005).

DCs can be found in the blood, in many different tissues and organs as well as in the lymph. In order to move around, these cells make use of different receptor-ligand interactions such as CCR2-CCL2, CCR5-CCL5 or CCR6-CCL20 for migration in non-lymphatic tissues as immature DCs and CCR7-CCL19/CCL21 for migrating in the lymphatics following maturation (Alvarez et al., 2008). They thereby follow concentration gradients and enter tissues in a similar way as other leukocytes, that is by tethering, rolling, adhesion and diapedesis that is dependent on different integrins as well as E- and P-selectin expression on the endothelial cells (Alvarez et al., 2008). Motile cells (such as skin DCs and Langerhans cells) are able to migrate from their original tissue via the lymphatic system to the draining lymph nodes, whereas the sessile cells stay in one place during their lifespan. To perform this migration, DCs first have to encounter a mobilization signal that induces their maturation, upregulate CCR7 and detach from the tissue by the disruption of E-cadherin junctions (Jiang et al., 2007). This is followed by their gradient-directed migration through the interstitium whereby DCs upregulate proteolytic enzymes such as matrix metalloproteinases (MMPs) in order to move through areas with high amounts of extracellular matrix proteins (Ratzinger et al., 2002; Weber et al., 2013). The final steps of this migration are the entering of afferent lymphatic vessels, usually at the lymphatic capillaries, followed by the semi-passive transport to the lymph nodes (Pflicke and Sixt, 2009).

Dendritic cells can exist in different maturation or activation stati and are therefore considered either immature or mature/activated. However, as with macrophage polarization, this is not a black and white system but rather a continuum. Both extremes differ not only in their phenotype, but also in their function. While immature DCs are very competent in endocytosis, they are not very good in antigen presentation due to their lack or low expression of MHC class I, MHC class II as well as the co-stimulatory receptors such as CD80, CD83 or

CD86. In contrast, once matured by exposure to microbial compounds or inflammatory cytokines, mature DCs have a very poor endocytic capability but excel at antigen presentation, as they express large amounts of MHC and co-stimulatory receptor molecules (Banchereau and Steinman, 1998; Clark et al., 2019; Münz et al., 2005; Worbs et al., 2017).

Furthermore, also the expression pattern of chemokine receptors is altered during maturation. Looking at the function of dendritic cells, these changes make a lot of sense. Immature cells in the periphery can take up bacterial or viral components, start their maturation and express the chemokine receptor CCR7. The expression of this receptor allows the cells to follow gradients of CCL19 and CCL21, thereby permitting them to enter terminal lymphatic vessels and follow the afferent lymphatics to the draining lymph nodes (Förster et al., 2008; Ohl et al., 2004). Once in the lymph node, the matured cells express high levels of MHC and therefore efficiently present their processed antigen to other immune cells such as T-cells as well as local dendritic cells. Additionally, DCs are also important when it comes to limiting autoimmune reactions as they can present self-antigens and thereby tolerize T cells (Banchereau and Steinman, 1998).

Another distinction of peripheral DCs was introduced after it had been discovered that a cell type described in 1958 and named plasmacytoid T-cells might actually belong to the myelo-monocytic lineage due to their expression of CD31, CD36 and CD68 (Facchetti et al., 1988). Found at low levels in the blood, these cells were at first called plasmacytoid monocytes by some researchers, until finally new evidence enforced their renaming to plasmacytoid dendritic cells (pDCs) (Galibert et al., 2001). pDCs therefore form a subgroup of peripheral DCs together with (myeloid) conventional/classical DCs (cDCs). Interestingly, it was not until 2001 that pDCs were found in mice (Asselin-Paturel et al., 2001; Björck, 2001; Nakano et al., 2001). Morphological, pDCs resemble plasma cells as they have an eccentric nucleus and a pronounced Golgi apparatus. Distinct features of pDCs are the fact that they fully develop in the bone marrow before they emigrate (in contrast to cDCs that already do this at a precursor state) and that they produce vast amounts of type I interferons (Gilliet et al., 2008). This makes them particularly good in dealing with viral infections, facilitated by their expression of TLR7 and TLR9, and also allows them to activate NK cells (Bao and Liu, 2013). However, in contrast to the myeloid cDCs, they are rather poor antigen presenters. Overall, human and mouse pDCs are remarkably similar. Nevertheless, while human pDCs are for example devoid of CD11c and thereby differ from their cDC counterpart, mouse pDCs express this transmembrane protein (Hochrein et al., 2002; Macal et al., 2012).

cDCs themselves also do not form one uniform population but can be further sub-grouped. Based on the expression of different transcription factors such as

interferon regulatory factors (IRF) 4 and 8 as well as results from gene expression studies, two different subtypes cDC1 (CD141$^+$ in humans and CD8a$^+$/CD103$^+$ in mouse) and cDC2 (CD1c$^+$ in humans and CD11b$^+$ in mouse) have been defined (Collin and Bigley, 2018; Guilliams et al., 2016; MacDonald et al., 2002; Merad et al., 2013; Mildner and Jung, 2014; Villani et al., 2017; Ziegler-Heitbrock et al., 2010). Naturally, these cells also differ in their functions and while cDC1s are better in stimulating CD8 T-cells, cDC2s are more adapt in stimulating responses of CD4$^+$ T cells (Wculek et al., 2020). While cDC1s and cDC2s are rather comparable in the murine and human system, the phenotype depends also on the tissue environment and therefore numerous closely related subtypes are possible (Collin and Bigley, 2018). As an example, both cDC1s and cDC2s can have a migratory or resident phenotype (Anderson et al., 2018). This, together with the realization that monocytes, macrophages and dendritic cells might not be as distinct as previously thought, promoted the introduction of a new unified nomenclature (Guilliams et al., 2014). Here, the authors suggest that cells of the mononuclear phagocyte system should be grouped by their ontogeny and only then subgrouped by phenotype, function and location. This suggestion introduced some big changes, as for example Langerhans cells would be classified as macrophages. It therefore remains to be seen to what extent these guidelines are implemented over time. Furthermore, established classifications might need to be adjusted and expanded due to new findings from single cell sequencing that allow for a broader and deeper understanding of the cell fate, some of which have already led to new knowledge about DC progenitors (Papalexi and Satija, 2018; Schlitzer et al., 2015). As an example, by applying single cell sequencing two subtypes of cDC2 cells, named cDC2A and cDC2B, have been defined thereby supporting earlier publications that had reported heterogeneity within this population (Brown et al., 2019; Kumamoto et al., 2009; Lewis et al., 2011). Similarly, another study that relied on single cell sequencing reported six different types of DCs in human blood (DC1 to DC6) instead of only pDCs and cDCs (Villani et al., 2017).

2.1.2 The adaptive immune system

In contrast to the more general and non-specific innate immune system, the hallmarks of the adaptive immune system are its specificity, its memory and its ability to discriminate 'self' and 'non-self'. This enables the targeting of distinct pathogens in a more specific, precise manner and allows their removal even though the innate immune system might not have been able to do so. However, the adaptive immune system is by no means a replacement of the innate system. Both systems supplement and even depend on each other. Due to the way how it functions, the adaptive immune system is relatively slow compared to the innate

immune system as instead of hours it rather takes days to mount a sufficient immune response. Nevertheless, the time it takes is well invested as the resulting response is specifically targeted to the invading pathogen and can even prevent future infections due to the formation of immunological memory. This works through an interplay of different immune cells from the innate and the adaptive immune system. Two of the most important cell types that belong to the adaptive immune system are the lymphoid derived B and T cells.

2.1.2.1   B cells

B cells originate and mature in the bone marrow and are named after the bursa of Fabricius, an organ in chickens where they had been discovered. They are quite versatile as they take on a variety of functions. Their best-known feature is their ability to produce antibodies, but additionally they are also capable of secreting cytokines and presenting antigens to T cells. Each B cell expresses a unique B-cell receptor on their surface that can recognize a specific antigen, and over the course of a lifetime millions of different antigens can be detected this way. This is an astonishing feat from B cells (and similarly also from T cells) as there is only a limited number of genes available. The generation of B-cell receptor diversity is achieved by implementing splicing, recombination and random insertion of different gene segments (Merlo and Mandik-Nayak, 2013). In some cases, the recognition of antigen by the B-cell receptor is enough to promote B-cell activation and proliferation (Coutinho and Möller, 1975). However, in most cases the B cell still requires a second co-stimulatory signal before it becomes activated. This second signal is usually delivered by an activated T-helper cell that has encountered the same antigen. Once it has received both signals, the B cell becomes a lymphoblast, starts to proliferate and transforms into an effector B cell (also referred to as plasma cells). Plasma cells produce and secrete antibodies that are specific for the original antigen that had bound to the B-cell receptor and triggered the proliferation (LeBien and Tedder, 2008). These antibodies therefore represent a secreted form of the B-cell receptor and are also called immunoglobulins (Ig). Antibodies can occur in five different forms named IgA, IgD, IgE, IgG and IgM. After being produced, they can be found in the extracellular fluids, thereby forming the humoral immunity. They bind specifically to their antigen on the invading pathogen and thereby assist in its clearance by different mechanisms. Their binding can activate the complement system, trigger cytotoxicity, opsonize the pathogen to promote its phagocytosis, neutralize it by preventing its adhesion to cells and membranes or simply by clumping it (agglutination). As with many other cells types, also B cells exist as different subtypes such as B1 cells in the peritoneum or as regulatory B cells (LeBien and Tedder, 2008; Mizoguchi and Bhan, 2006). Additionally, after the encounter

with their specific antigen some B cells become memory B cells that survive for many years and allow for a fast and efficient response if the same antigen should be encountered again.

2.1.2.2   T cells

Like B cells, also T cells are originally produced in the bone marrow and like B cells, they also express a receptor, here called the T cell receptor, that is related to immunoglobulin and specific for one antigen. However, they still differ tremendously from B cells. The precursors of T cells do not stay in the bone marrow but migrate out and continue their development in the thymus. Their main function is also not to take part in the humoral immune response by secreting antibodies, but they are rather important players in the cell-mediated immune response. In addition, T cells react only to processed antigen that is presented to them via MHC (major histocompatibility complex) molecules, while B cells react directly to unprocessed antigen. Based on the mutually exclusive expression of two different molecules on their surface, T cells can be classified into $CD4^+$ and $CD8^+$ cells, respectively. However, functionally, T cells can be distributed into at least three main subsets: $CD8^+$ cytotoxic T cells that can kill virus-infected cells, $CD4^+$ helper T cells that support other immune cells by activating them and $CD4^+$ regulatory T cells that can dampen the activity of other cells and shut down an immune response. Cytotoxic T cells are CD8-positive cells that are able to recognize processed peptides presented via MHC class I molecules (Mizoguchi and Bhan, 2006). As MHC class I molecules are expressed on all nucleated cells, they usually present peptides that have been produced within the cell itself, thereby presenting the body's own molecules. This expression of self-peptides has no negative effect on the cells as T cells have become tolerant during their development for these peptides and recognize them as "friendly" (Adamopoulou et al., 2013). However, if the cell becomes infected with a virus, the MHC class I molecules present viral peptides on its surface, thereby triggering primed $CD8^+$ T cells to destroy the cell (Harty et al., 2000).

The second group of T cells, helper T cells (T_H cells), express CD4 on their surface and interact with peptides presented on MHC class II molecules. In contrast to MHC class I molecules, class II molecules are only expressed on antigen-presenting immune cells, such as macrophages, dendritic cells and B cells. Furthermore, they are not loaded with peptides produced within the cell itself, but they are presenting parts of proteins or pathogens that have been phagocytosed and lysed by these immune cells (Punt, 2013). T_H cells can be divided into at least four different groups: T_H1, T_H2, T_H17 and follicular T helper cells (Geginat et al., 2014). T_H1 cells are important for the cell-mediated immunity as they secrete cytokines such as IFN-γ and TNF-α and thereby are able to stimulate macrophages. In

contrast, T_H2 cells are more involved with humoral immunity as they secrete for example IL-4 and IL-5, are thereby important stimulators of B cells and protect against extracellular pathogens. The third type, T_H17 cells, secrete IL-17, are able to recruit neutrophils and are important for the immune response against fungi and other extracellular pathogens. Finally, follicular T helper cells secrete IL-21 and assist B cells in their response (Geginat et al., 2014; Punt, 2013).

Finally, the third main group of T cells are the CD4^{+} regulatory T cells. These cells, that have previously been called suppressor cells, can regulate the response of other T cell populations. They do this by direct cell-contact or secreting cytokines such as TGF-β and IL-10 and they are important in limiting autoimmune activity (Geginat et al., 2014; Punt, 2013).

2.2 The vascular system

In order to function, the bodies of vertebrates require a constant supply with resources such as nutrients and oxygen. However, it is not enough to ingest them as they also need to be distributed throughout the body. While in small animals such as *Caenorhabditis elegans* this is possible by diffusion, vertebrates are too big for this and therefore require a specific system that fulfills this task. This system is called the vascular system and consists of two different interconnected circuits, the blood and the lymphatic vascular system (Carmeliet, 2005) (Figure 2). Both systems have in common, that they are somewhat permeable, thereby allowing for an exchange of their content with the extravascular tissue compartments. The blood vascular system consists of arteries and veins that transport blood, cells, nutrients, oxygen as well as various macromolecules. They are connected by capillaries which, due to their small size and lack of an investing smooth muscle layer, allow for an easy exchange of their content. The flow in these vessels is controlled by the pumping of the heart. In contrast, the lymphatic vascular system consists of lymphatic vessels, lymph nodes and, instead of blood, transports lymph. This transport is not relying on a special organ, but works somewhat indirectly due to muscle movement (Zawieja, 2009). Much like the blood vascular system, also the lymphatic vascular system fulfills multiple roles. Due to the blood pressure, blood plasma is constantly leaking out of the blood vessels into the adjacent interstitial space, amounting in humans to around two liters of fluid every day. This is where the lymphatic system comes into play. It works as a drainage system that takes up the leaked liquids, transports them past lymph nodes to the thoracic or the right lymphatic duct where they will be returned to the blood circulation. Additionally, the lymphatic system transports dietary lipids that had been absorbed in the gut and it is also an essential part of the immune system (Aspelund et al., 2016; Oliver and Alitalo, 2005; Zawieja, 2009).
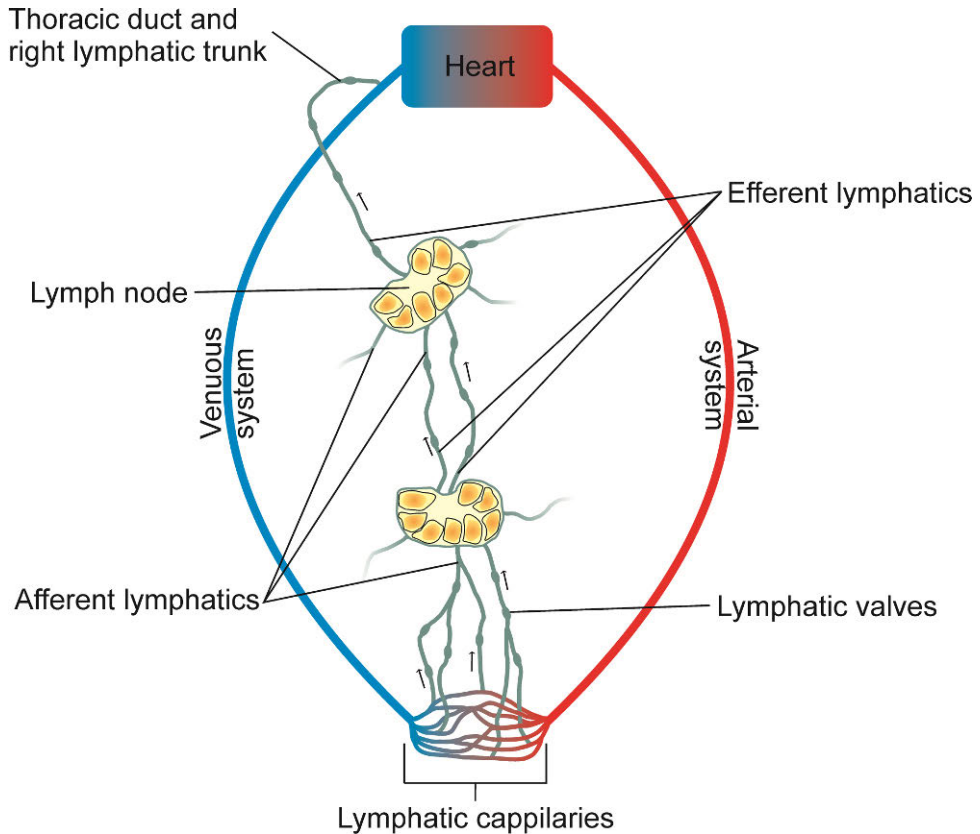
Figure 2. Simplified overview of the vascular system. Blood is pumped by the heart through the arterial and venous systems to supply the body with oxygen and nutrients. This is facilitated by the permeability of the blood vessels, but at the same time also leads to leakage of blood plasma into the vessel-periphery. Here, the lymphatic capillaries collect this fluid, cells and macromolecules and unidirectionally transport them via afferent lymphatic vessels to draining lymph nodes. In the lymph nodes, the lymph fluid is filtered and an adaptive immune response can be initiated. After the lymph exits a lymph node it can be transported via efferent lymphatic vessels to additional lymph nodes by means of lymphatic vessels that are then again termed 'afferent', until it finally re-enters the venous circulation via the thoracic duct or the right lymphatic trunk. Adapted from Aspelund et al., 2016.

Both the blood and most of the lymphatic vessels are surrounded by connective tissue and smooth muscle cells. Moving further towards the inside of the vessel, this is followed by a basal lamina that is lined by a single layer of endothelial cells (EC, blood endothelial cells (BEC) and lymphatic endothelial cells (LEC), respectively) which face the lumen of the vessels. Although both BECs and LECs express CD31 and CD34, and for a long time no specific marker to separate them had been available, there are big differences between the two ECs (Pusztaszeri et al., 2006). In fact, even within the same type of endothelium, the ECs can differ quite a bit. For the

BECs, which in general are positive for PAL-E and CD105 (Endoglin), the largest distinction can be made between arterial (i.e. cells in the blood vessels that come from the heart) and venuous cells (i.e. cells in the blood vessels that lead to the heart) (Hirakawa et al., 2003; Keuschnigg et al., 2009). Structurally, arterial ECs are thicker with an elongated shape compared to the short venular ECs. This is not surprising, as arterial cells have to withstand much higher pressures than venular cells due to the sheer stress in arteries being around ten times higher and reaching 10-40 dynes/cm^2 (dela Paz and D'Amore, 2009). One of the major difference in their function is that arterial cells are important for controlling the vascular tone, while venular cells are essential for the trafficking of leukocytes (dela Paz and D'Amore, 2009). However, also besides the arterial and venular differences BECs can be found in three different phenotypes, namely 'Continuous, non-fenestrated', 'Continuous, fenestrated' and 'Discontinuous, sinusoidal'. Continuous hereby indicates that BECs are tightly connected to each other without any big gaps, while discontinuous ECs have gaps between them. Similarly, fenestrated ECs have round openings containing a diaphragm acting as a filter. These fenestrae connect their apical and basal side and are used for the exchange of water and other small molecules. In contrast, non-fenestrated ECs rely on transendothelial channels or caveolae for the exchange (dela Paz and D'Amore, 2009).

Similarly to arterial and venular BECs, for the Lyve-1, Prox-1 and Podoplanin positive lymphatic endothelial cells the largest difference in the endothelial cell type can be found between the afferent lymphatics (i.e. the lymphatic vessels that drain to a lymph node) and the efferent lymphatics (i.e. the lymphatic vessels that drain from a lymph node) (Hunter et al., 2016; Johnson and Jackson, 2008). In sheep, it has been measured that the flow-rate of lymph around one skin-draining lymph node in the afferent lymphatics is low compared to the efferent lymphatics, as it reaches about 2-5x10^6 cells/h in the resting state and 5-10x10^6 cells/h in the stimulated state (HAIG et al., 1999). In contrast, in the efferent lymphatics these values are at least 10 fold higher and amount to around 2-5x10^7 cells/h and 1-5x10^8 cells/h in the resting and stimulated state, respectively (HAIG et al., 1999). This drastic difference can be explained by the fact, that more than 90% of the cells that can be found in the efferent lymphatics had entered the lymph node from the blood stream via high endothelial venules (HEV) and did not arrive via the afferent lymphatics.

Interestingly, although it is often depicted that only one efferent lymphatic vessel is exiting a lymph node, also multiple efferent vessels are possible (Pan et al., 2010). In addition to the difference in the cell amounts that can be found within these vessels, also the cell composition is different. On the afferent side, around 10% of the cells are dendritic cells, a cell population that is absent on the efferent side. Instead, the efferent side has a 20% higher proportion of B cells (HAIG et al., 1999). This is not surprising when one considers the role these vessels and the

lymph nodes play in immune surveillance as well as in immune reactions. The lymph nodes hereby act as a gateway, allowing leukocytes to enter from the afferent side, but only allow lymphocytes to exit again. Cells such as DCs that have taken up antigen in the periphery, matured and thereafter migrated via the afferent lymphatics to the lymph node, will stay there and present their processed antigen until they die. This allows for an efficient screening and the activation of T-cells that recognize this specific antigen. Once they encounter it, they start to proliferate and a massive efflux of cells from the lymph node can be detected. Therefore, much like in the blood vasculature, also the differences in the lymphatic vasculature are due to their different functions. This is also reflected by the expression of certain molecules that can exclusively be found on only one part of the lymphatics. In mice, CD169 (Siglec-1) and CD204 (MSR1) could only be found on the afferent side, while endomucin was limited to the efferent side (Iftakhar-E-Khuda et al., 2016).

Furthermore, in both humans and mice the expression of the ectonucleotidase CD73 (NT5E) is limited to afferent lymphatic endothelial cells (Ålgars et al., 2011). While the afferent lymphatics have to collect, drain and transport fluid and cells from the periphery to the draining lymph nodes, the efferent lymphatics facilitate a quick and efficient transport of filtered fluid and cells to return them to the blood circulation. At the lymphatic periphery, in the capillaries, the presence of specialized oak-leaf shaped endothelial cells allows interstitial fluid to enter. These overlapping cells, or ECs, enable the fluid (and cells) to access the lymphatic vessels, but prevent it from flowing back. Interestingly, this entry of cells is independent of proteases as inhibiting them did not alter cell migration – contrasting experiments done with blood endothelium where cell exit depends on protease activity (Butcher and Picker, 1996; Ley et al., 2007; Pflicke and Sixt, 2009). From this point on, the interstitial fluid is called lymph and is transported along the afferent lymphatic capillaries into lymphatic vessels. As lymph fluid is drained from interstitial spaces all over the body, it does not only contain nutrients and fats, but can also transport pathogens or antigens that were present in the original tissues. In the lymphatic vessels, the presence of valves prevents the back-flow of the lymph and forces it to flow in only one direction – towards the draining lymph nodes. Lymph nodes in humans and mice fulfill similar roles, however, while humans have several hundreds of them, mice only have 22 (Van den Broeck et al., 2006). They are distributed all over the body, filter lymph and are mainly populated by immune cells such as B cells, T cells, macrophages and dendritic cells. Lymph nodes are therefore crucial for adaptive immune responses. Structurally, lymph nodes are encapsulated organs with a peripheral cortex consisting of an outer and inner cortex and a centrally located medulla, all of which contain heterogeneous populations of LECs that express partly different marker

molecules compared to those found in the periphery (Das et al., 2013; Takeda et al., 2019). Within the cortex are lymphatic follicles that can contain germinal centers, areas of intense B cell proliferation during an immunological reaction. Once the lymph arrives in the draining lymph nodes, it enters into the subcapsular space below the capsule, where it is filtered and screened by local macrophages and dendritic cells before it continues via lymphatic sinuses to the cortex to encounter T cells (HAIG et al., 1999; Jalkanen and Salmi, 2020). The final station in the lymph node is the medullary sinus with its abundance of B and plasma cells before the lymph exits the node via efferent lymphatic vessels. These then either drain into another lymph node (and thereby become afferent vessels themselves) or return the lymph to the blood circulation in the right lymphatic or thoracic duct.

Interestingly, the lymphatic vessels are more than just a way to transport immune cells. It has been shown that LECs in the lymph node interact with different immune cells and are capable of antigen presentation and activation of CD8$^+$ T cells via MHC-class I, while at the same time limiting these responses due to their expression of PD-L1 (Berendam et al., 2019). Furthermore, these LECs also could express MHC-class II molecules and although they were not able to present antigen directly to CD4$^+$ T cells, they could transfer it to DCs which then in turn could interact with CD4$^+$ T cells (Rouhani et al., 2015). A similar transfer is also possible in the opposite direction as it has been shown that LECs can accept formed complexes of MHC-class II and self-antigens from DCs and then present those to CD4$^+$ cells (Dubrot et al., 2014). It even has been shown, that LECs are capable of archiving antigens and thereby forming a reservoir supporting immunological memory (Kedl et al., 2017; Tamburini et al., 2014). Besides the transfer of antigen, the contact with DCs additionally induced proliferation in the LECs, therefore indicating a reciprocal interaction (Webster et al., 2006). Similarly, also macrophages and B cells could induce proliferation of lymph node LECs, while T cells seem to inhibit it (Angeli et al., 2006; Kataru et al., 2009, 2011). Finally, also LECs can influence the survival and proliferation of immune cells as has been shown for their interaction with T cells (Iolyeva et al., 2013; Mendoza et al., 2017; Nörder et al., 2012).

2.3   CD73

CD73, or ecto-5'-nucleotidase, is an ectoenzyme which is most widely known for its dephosphorylating ability that converts AMP into adenosine and phosphate. CD73 is encoded by the NT5E gene and it consists of a N-terminal domain harboring a metal-ion binding site and is linked to the C-terminal domain by an alpha helix (Knapp et al., 2012). The C-terminal domain is smaller and contains the substrate binding and the dimerization sites. Due to the flexibility of the alpha

helix, the molecule is able to do rotations of up to 114° and together with the interchain movement can form an 'open' and a 'closed' conformation (Knapp et al., 2012). These conformation changes are necessary for its enzymatic activity, allowing substrate to bind during the 'open' state and facilitating the enzymatic reaction in the 'closed' state by bringing the N- and C-terminal domains in proximity to each other (Figure 3).
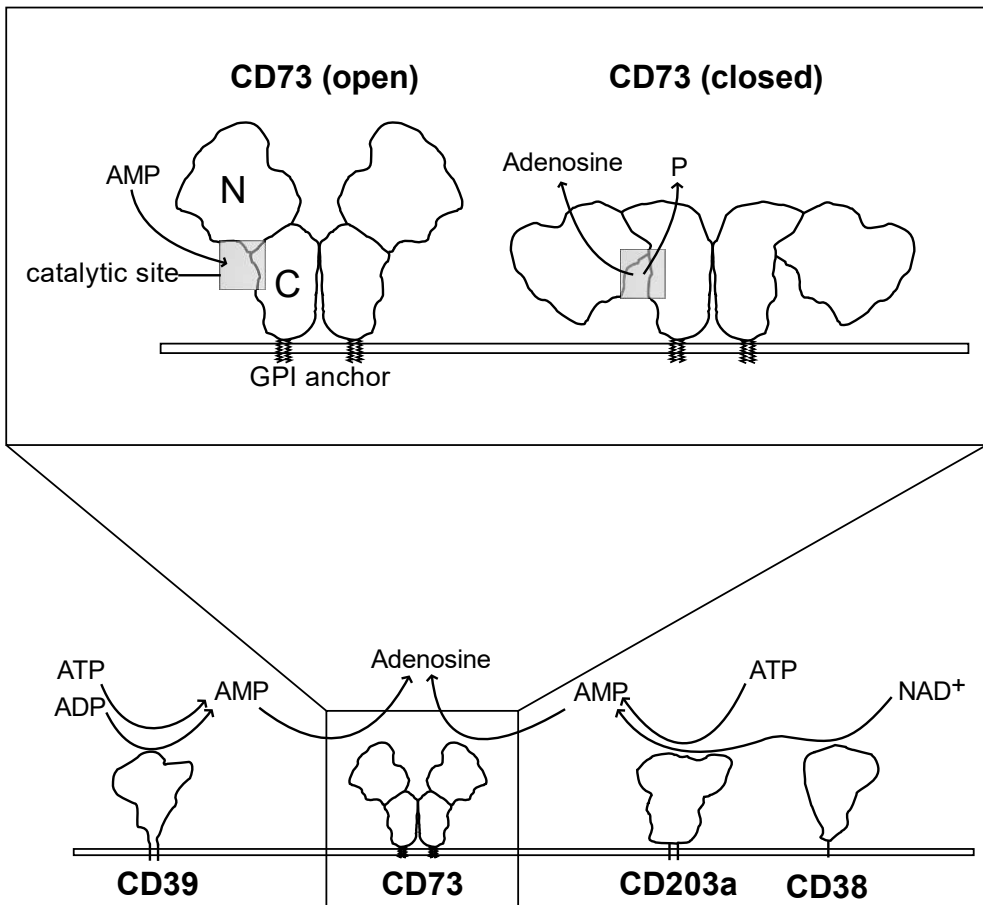
Figure 3. (Top) The homodimer CD73 is anchored to the membrane by a GPI anchor and has two distinct conformations, open and closed. In the open form AMP can bind to the catalytic site while in the closed form the dephosphorylation of AMP to adenosine and phosphate can take place. (Bottom) AMP required for the activity of CD73 can be provided by different purinergic ectoenzymes that metabolize ATP (CD39 and CD203a), ADP (CD39) or NAD+ (CD38/CD203a). Adapted from (Knapp et al., 2012 and Schneider et al., 2019).

The CD73 molecule has been found in a secreted as well as in a membrane-bound form and due to its four N-glycosylation sites (in human and mouse), its molecular

weight varies between 60 and 80 kDa, depending on the tissue (Zimmermann, 1992). When bound to a membrane, CD73 is anchored by glycosylphosphatidylinositol (GPI) in either a phospholipase C sensitive or resistant form (Klemens et al., 1990; Vogel et al., 1992). When shed, CD73 can additionally affect adenosine levels at greater distances from its original location, thereby allowing for a more comprehensive immunoregulatory effect. This effect is enhanced by the fact, that phospholipase C cleaved CD73 has a higher ectonucleotidase activity than its membrane-bound counterpart (LEHTO and SHAROM, 1998; Low and Finean, 1978). Furthermore, the shedding has also been considered as a mechanism that prevents a premature deactivation of the original host cell by 'self-regulation'. As an example, on T cells CD73 is expressed to keep them in a resting state (Cekic et al., 2013). Once the cells get activated, CD73 is replaced by CD39 in order to keep adenosine-levels in the direct environment low and allow for a strong T-cell activation. CD73 that is released during this process, is then thought to reach activated cells in other tissues, restrict their activation and facilitate the resolution of inflammation (Schneider et al., 2019). To be enzymatically active, CD73 forms homodimers that together make up the catalytic site, where extracellular AMP can be dephosphorylated to adenosine and inorganic phosphate (Ogata et al., 1990).

CD73 can be found to be abundantly expressed on subsets of T- and B-cells, epithelial cells, smooth muscle cells, endothelial cells and at more sporadic and lower expression levels on numerous other cells and cell types, such as NK cells or monocytes/macrophages (Stagg and Smyth, 2010; Yegutkin et al., 2015). It is, however, important to keep in mind that the expression of CD73 is highly species- and tissue-dependent (Minor et al., 2019). This can, for example, be seen on Treg cells, which in the mouse commonly express CD73 while in humans only a minor portion of these cells expresses it on their surface (Deaglio et al., 2007; Mandapathil et al., 2010).

Furthermore, the expression-levels of CD73 are not fixed but can vary depending on external stimuli and the cell environment. It has been shown that hypoxia, IFN-β, TNF-α, TGF-β, retinoic acid as well as vitamin D are able to induce its expression (Francois et al., 2015; Mann et al., 2015; Regateiro et al., 2011; Tóth et al., 2013). This alteration in CD73 expression can have a profound impact on cell functions, as for example Th17 cells that express CD73 promote tumor growth, while CD73-negative Th17 cells possess anti-tumor functions (Chatterjee et al., 2014).

In order to be enzymatically active, CD73 requires its substrate AMP. AMP is derived from catalytic actions of other molecules such as CD39 (NTPDase1) that dephosphorylates ATP and ADP or CD38 (NPP1) and CD203a (ENPP1) that together convert NAD^+ to AMP (Horenstein et al., 2013; Schneider et al., 2019; Yegutkin, 2014; Zimmermann et al., 2012). In addition, CD203a can also generate

AMP directly from ATP. These substrates are often released from cells as a stress signal during damage or cell death and therefore act as danger-associated molecular patterns (DAMPs) triggering pro-inflammatory reactions. As an example, it has been shown that ATP is involved in the chemotaxis as well as the cytokine production of inflammatory cells (Iwata et al., 2016; Kronlage et al., 2010). The dephosphorylation of these molecules into anti-inflammatory adenosine thereby marks one of the most important features of CD73. Together with its abundant expression, CD73 (in collaboration with e.g. CD39) is therefore able to transform a cellular microenvironment from pro- to anti-inflammatory, often creating an adenosine halo in its vicinity. The produced adenosine can then be bound by different G-protein coupled P1 receptors (A1, A2A, A2B, A3) or converted to inosine (Fredholm et al., 2011; Haskó and Cronstein, 2013). The four P1 receptors differ in their affinity for adenosine in that A1- and A2A-receptors have a high affinity, while the affinity of A2B- and A3-receptors is low. The downstream effects differ, depending on what receptor is engaged, but overall adenosine reduces the release of pro-inflammatory cytokines while at the same time promoting anti-inflammatory cytokines (Haskó and Pacher, 2012; Haskó et al., 2007; Koscsó et al., 2012). Furthermore, adenosine inhibits Th17 cells and induces Treg cells (Bao et al., 2016; Ohta et al., 2012). It is noteworthy that while bacterial CD73 can additionally hydrolyze ATP or ADP, mammalian CD73 cannot and both of these molecules act as competitive inhibitors to its activity (Yegutkin et al., 2001).

Interestingly, while some cell types can co-express multiple proteins responsible for this cascade, more often the generation of adenosine is achieved through paracrine interactions of different cells or vesicles where each cell partner only expresses one part of the cascade (Clayton et al., 2011; Deaglio et al., 2007; Schuler et al., 2014). This allows for a more precise control of immune responses. In addition to its anti-inflammatory role, adenosine also affects the differentiation, growth or migration in a wide variety of cells (Antonioli et al., 2013a; Chen et al., 2006; Haskó, 2004; Schwaninger et al., 2002). Furthermore, adenosine generated by CD73 is essential for maintaining the barrier function of vascular endothelium (Thompson et al., 2004). This is especially prominent under hypoxic conditions in CD73 KO animals, as hypoxia-inducible transcription factors (HIF) normally elevate CD73 expression. As this cannot happen in CD73 KO animals, reduced adenosine levels and vascular leakage are the consequence (Thompson et al., 2004). However, this does not seem to apply universally for all endothelia. In the brain, CD73 derived adenosine actually supports the permeability and is needed for immune cells to cross the blood brain barrier. This effect is so dramatic, that CD73 KO animals are protected from EAE, an animal model of multiple sclerosis, as autoreactive immune cells are not able to enter the brain to destroy the myeloid sheets of the nerve cells (Bynoe et al., 2015; Mills et al., 2008, 2012).

Similarly, also between blood- and lymphatic endothelium the role of CD73 is quite different. On blood-endothelium CD73 enhances the barrier function, sprouting of the endothelial cells and also plays a role in the adhesion and transmigration of lymphocytes (Airas et al., 1995, 2014; Yegutkin et al., 2015). It has been shown that adhering lymphocytes block the enzymatic activity of CD73, thereby lowering local adenosine levels and making the endothelium less tight, allowing for an easier transmigration of cells (Yegutkin et al., 2015).

In contrast, the role of CD73 on lymphatic endothelial cells has not been studied as extensively. In part, this may be because CD73 is only expressed on afferent lymphatics but not on the efferent part (Ålgars et al., 2011). Additionally, although its expression on human afferent lymphatic endothelium is high, in mice it is sporadic (Ålgars et al., 2011). Nevertheless, it has been found that adenine nucleotides (such as adenosine) do not affect the barrier function nor the lymph angiogenesis (Yegutkin et al., 2015). Furthermore, unlike in blood endothelium, CD73 on lymphatic endothelium does not affect the migration of lymphocytes or dendritic cells. Instead, lymphocyte CD73 allows for an efficient migration to draining lymph nodes as this migration is reduced by 50% in its absence (Ålgars et al., 2011).

One study area that in recent years has gained a lot of attention involving CD73 and the purinergic signaling system in general is cancer. As cancer tissue grows, its core becomes necrotic and cells die. In addition, due to the rapid metabolism of cancer cells, their environment is often hypoxic. Both of these effects lead to drastically elevated levels of ATP (a 1000-fold increase from almost non-detectable levels to micromolar-levels) (Pellegatti et al., 2008). While this should be beneficial for fighting the cancer, as elevated levels of ATP stimulate the immune system, due to the presence of CD73 and CD39 on most cancers the opposite is actually the case. The pro-inflammatory amounts of ATP get converted into immunosuppressive adenosine that can reach micro-molar concentrations and activate for example A2B receptors, creating a tumor-favoring environment that limits the activation of the immune system (Allard et al., 2016a; Antonioli et al., 2013b; Blay et al., 1997). Two cell types that can be involved in this are macrophages and dendritic cells. Macrophages can, when exposed to adenosine, produce IL-4 and IL-10 and thereby limit the antitumoral response, whereas DCs can become angiogenic and immune-suppressive (Kumar, 2013; Novitskiy et al., 2008). In addition, this has been shown in studies implementing CD39- or CD73-deficient animals or by using inhibitors for these molecules where this intervention drastically limited tumor growth and development (Perrot et al., 2019; Sun et al., 2010). Targeting adenosine, CD73 and its related molecules has therefore an enormous potential for clinical applications. This is currently investigated in multiple clinical trials that use different anti-CD73 antibodies or target adenosine receptors as a potential anti-cancer treatment (Allard et al., 2014; Barnhart et al., 2016; Lu et al., 2016; Perrot et al., 2019). Even so, the

outcome of such interventions can vary depending on the cancer type and tumor model. In addition, while often the CD73 expression on immune cells such as Treg cells plays an important role, also CD73 expression on endothelial cells has been shown to be involved by processes such as reducing ICAM-1 expression and limiting T cell homing (Stagg et al., 2012; Wang et al., 2011).

Interestingly, although CD73 expression has been associated with an unfavorable clinical outcome in a multitude of cancers, in some cancer types its expression actually indicates a good prognosis (Allard et al., 2016b; Antonioli et al., 2016; Bowser et al., 2016; Supernat et al., 2012). CD73 therefore seems to perform rather diverse roles that depend on the cancer model. It can promote the growth and migration of malignant cells (Antonioli et al., 2013a; Eltzschig et al., 2012), but also have opposite effects, such as supporting cell apoptosis (Shirali et al., 2013; WANG and REN, 2006). Similarly, also in other diseases the expression of CD73 can have different effects. In acute respiratory distress syndrome (ARDS) elevated CD73 levels seem to be beneficial, although more studies are required to solidify these findings (Bellingan et al., 2014; Ranieri et al., 2020). In contrast, in humans (but not in mice) the deficiency for CD73 can lead to the calcification of small joints and the vasculature, arteriomegaly as well as tortuosity (Joolharzadeh and St. Hilaire, 2019). An overview of different phenotypes that are caused due to CD73-deficiency can be seen in Figure 4.

Finally, while most research is focusing on the adenosine-producing aspect of CD73, it has been shown that this molecule can additionally exert enzymatically-independent functions. Amongst these are the promotion of migratory and proliferatory effects whereby CD73 can act as a membrane receptor capable of interacting with fibronectin, laminin and Tenascin C (Olmo et al., 1992; Sadej et al., 2008). As an example, CD73 supports the adhesion of melanoma cells to Tenascin C (Sadej and Skladanowski, 2012). Furthermore, CD73 can act as a co-stimulator during T-cell activation, can support the phosphorylation of proteins and can protect against apoptosis (Airas et al., 1997a; Dianzani et al., 1993; Mikhailov et al., 2008; Resta and Thompson, 1997).

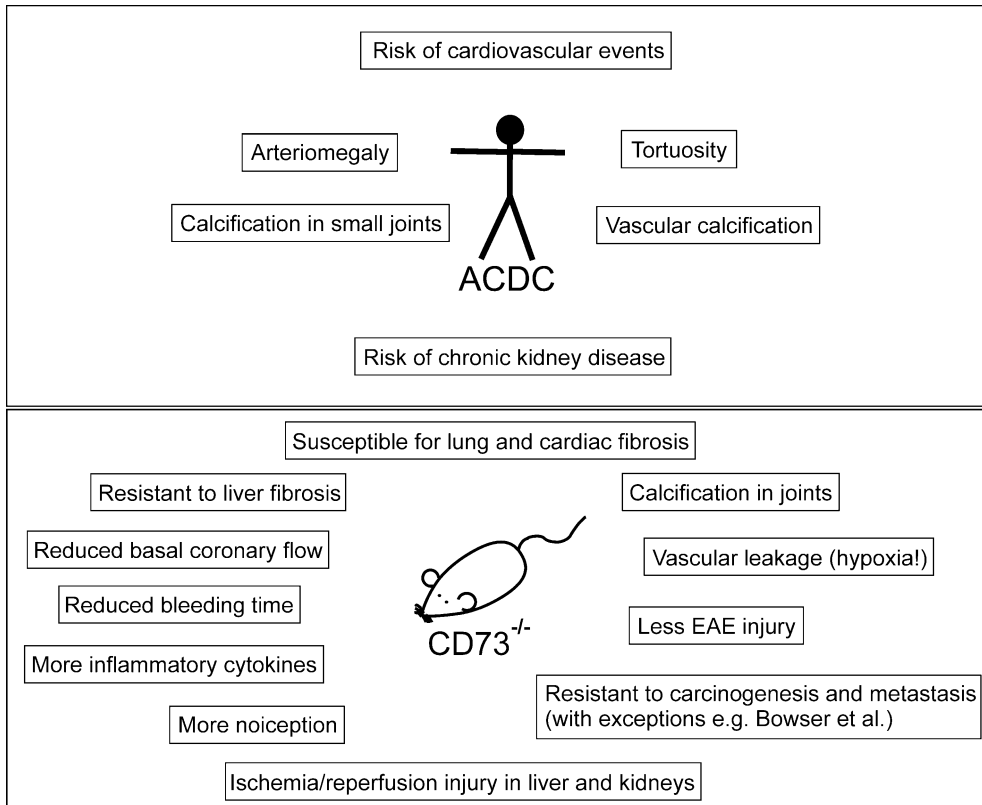**Figure 4.** Deficiency for CD73 has different effects in mice and humans. The phenotype of mice that are deficient for CD73 can be seen on the top, the phenotype of humans with a mutation in CD73 is shown on the bottom. ACDC=Arterial calcification due to deficiency of CD73, EAE=Experimental autoimmune encephalomyelitis. Adapted from (Minor et al. 2019, Joolharzadeh and St. Hilaire 2019 and Bowser et al. 2016)

3   Aims

In recent years, CD73 has received more and more attention when it became clear, that this ectonucleotidase is not only present on a multitude of tissues and cells, but that it also fulfills many different important roles in the body. First discovered for its enzymatic activity, it turned out that CD73 is involved in the regulation of tissue homeostasis while also being able to regulate immune responses. The interest in this molecule peaked when it became clear, that besides normal bodily functions, CD73 is also involved in the development of cancer, as well as in the immunological responses fighting it. However, because of its multi-faceted nature, the exact role that CD73 plays in different tissues is not always clear. This role seems to be dependent on many factors, such as the cytokine environment, the type of cells CD73 is expressed on, surrounding cells and many other aspects. It is therefore crucial to investigate the role of CD73 in different locations and situations meticulously in order to avoid jumping to wrong conclusions.

The aim of this study therefore was to shed light into two specific aspects of CD73:

I   To determine the expression and importance of CD73 in macrophage polarization in human and mouse cells.

II   To discover the role of CD73 on afferent lymphatic cells as well as determining what effect the blocking of CD73 with therapeutical antibodies has for the function of the blood- and lymphatic-vasculature.

4   Materials and Methods

The information described here is a brief selection of all materials and methods used in this study. It contains mainly materials and methods that were not included in the original publications and their supplements or a concise summary of included methods. A more detailed description can be found in the original publications and their supplements.

4.1   Antibodies

Table 1.   Summary of all antibodies used in the studies.

Antibody	Target Species	Isotype	Product #	Used in
anti-mouse IgG AF546	Mouse	Goat	Invitrogen A11030	II
anti-mouse IgG IRDye 800CW	Mouse	Donkey	Licor 926-32212	II
anti-mouse IgG1 AF488	Mouse	Goat IgG	Invitrogen A21121	I
anti-mouse IgG2b PE	Mouse	Goat IgG	SouthernBiotech 1090-09	I
anti-rabbit IgG AF546	Rabbit	Goat	Invitrogen A11035	II
anti-rabbit IgG FITC	Rabbit	Sheep	Sigma F7512	I
anti-rat IgG AF488	Rat	Donkey	Invitrogen A21208	II
AK1	-	Mouse IgG1	in house	I
CD1 PE	Human	Mouse IgG2a	LS Bio LS-C196193	II
CD1a AF700	Human	Mouse IgG1	Biolegend 300120	II
CD1a PerCP-Cy5.5	Human	Mouse IgG1	Biolegend 300130	II
CD5 PE	Mouse	Rat IgG2a	BD 553023	I
CD8a AF647	Mouse	Rat IgG2a	BD 557682	II
CD11b APC	Mouse	Rat IgG2b	BD 553312	I
CD11b APC-Cy7	Mouse	Rat IgG2b	BD 557657	II
CD11c APC	Human	Mouse IgG1	BD559877	II
CD11c BV421	Mouse	Hamster IgG1	BD 562782	II
CD11c FITC	Mouse	Hamster IgG	Biolegend 117306	II
CD11c PerCP-Cy5.5	Mouse	Hamster IgG1	BD 560584	II
CD14 APC	Human	Mouse IgG2a	BD 555399	II
CD14 APC	Human	Mouse IgG2a	BD 555399	II

Antibody	Target Species	Isotype	Product #	Used in
CD14 PE	Human	Mouse IgG1	Biolegend 325606	II
CD14 PB	Human	Mouse IgG2a	BD 558121	I
CD31 FITC	Human	Mouse IgG1	Beckman Coulter IM1431U	II
CD40 APC	Human	Mouse IgG1	Biolegend 334310	II
CD40 APC	Mouse	Rat IgG2a	BD 558695	II
CD40 BV510	Human	Mouse IgG1	BD 563456	II
CD40 PE-CF594	Mouse	Rat IgG2a	BD 562847	II
CD45 BV421	Human	Mouse IgG1	BD 563879	II
CD45 BV510	Human	Mouse IgG1	Biolegend 304036	II
CD45 PE	Human	Mouse IgG1	BD 555483	II
CD73 (rNu-9)	Rat	Rabbit IgG	Provided by Prof. Jean Sevigny, Laval University, Quebec, Canada (http://ectonucleotidases-ab.com)	II
CD73 (118)	Human	Mouse IgG2b	in-house	I, II
CD73 (4G4)	Human	Mouse IgG1	in-house	II
CD73 (AD2)	Human	Mouse IgG1	BD 550256	II
CD73 PE	Mouse	Rat IgG2a	BD 550741	I
CD73 PE-CF594	Human	Mouse IgG1	BD 562817	II
CD73 PerCP-Cy5.5	Human	Mouse IgG1	BD 561260	II
CD80 PE	Human	Mouse IgG1	BD 557227	II
CD80 PerCP-Cy5.5	Mouse	Hamster IgG2	BD 560526	II
CD83 BV421	Human	Mouse IgG1	BD 562630	II
CD83 BV421	Human	Mouse IgG1	Biolegend 305324	II
CD86 APC-R700	Mouse	Rat IgG2a	BD 565479	II
CD86 PE	Mouse	Rat IgG2a	BD 553629	II
CD86 PerCP-Cy5.5	Human	Mouse IgG1	BD 561129	II
CD103 PE	Mouse	Rat IgG2b	BD 557495	II
CD206	Human	Mouse IgG1	LifeSpan LS-C40886	I
CD206 AF488	Mouse	Rat IgG2a	AbD Serotec MCA2235A488	I
F4/80	Mouse	Rat IgG2a	Biolegend 123110	I
F4/80 FITC	Mouse	Rat IgG2b	AbD Serotec MCA497A488	I
FC-block	Mouse	Rat IgG2b	BD 553142	I
Fixable Viability Dye eFluor 780	-	-	eBioscience 65-0865-18	II
HLA Class I FITC	Human	Mouse IgG2a	Sigma F5662	II
HLA-DP,-DQ,-DR FITC	Human	Mouse IgG2a	BD 555558	II
HLA-DR PE-Cy7	Human	Mouse IgG2a	Biolegend 307616	II
ICAM-1	Human	Rabbit IgG	Santa Cruz sc-7891	II
ICAM-1 (CD54) AF488	Mouse	Rat IgG2b	Southern Biotech 1701-30	II
IgG	-	Hamster	BD 562919	II
IgG1	-	Mouse	BD 555746	II
IgG1 AF700	-	Mouse	BD 557882	II
IgG1 APC	-	Mouse	R&D IC002A	II

Antibody	Target Species	Isotype	Product #	Used in
IgG1 APC	-	Mouse	Biolegend 400120	II
IgG1 BV421	-	Mouse	Biolegend 400157	II
IgG1 BV421	-	Mouse	Biolegend 400158	II
IgG1 BV510	-	Mouse	BD 562946	II
IgG1 BV510	-	Mouse	Biolegend 400172	II
IgG1 FITC	-	Mouse	BD 554679	II
IgG1 PE	-	Mouse	BD 555749	II
IgG1 PE	-	Mouse	Biolegend 400114	II
IgG1 PE-CF594	-	Mouse	BD 562292	II
IgG1 PerCP-Cy5.5	-	Mouse	BD 552834	II
IgG1 PerCP-Cy5.5	-	Mouse	Biolegend 400150	II
IgG1 PerCP-Cy5.5	-	Hamster	BD 550763	II
IgG2 PerCP-Cy5.5	-	Hamster	BD 560562	II
IgG2a AF488	-	Rat	Biolegend 400525	I
IgG2a AF647	-	Rat	BD 557906	II
IgG2a APC	-	Mouse	BD 340473	II
IgG2a APC-R700	-	Rat	BD 564982	II
IgG2a FITC	-	Mouse	BD 553456	II
IgG2a PE	-	Rat	BD 553930	I
IgG2a PE	-	Rat	eBioscience 12-4321-81a	II
IgG2a PE-CF594	-	Rat	BD 562302	II
IgG2a PE-Cy7	-	Mouse	Biolegend 400232	II
IgG2a PB	-	Mouse	BD 558118	I
IgG2b	-	Mouse	Biolegend 401212	I, II
IgG2b APC	-	Rat	BD 553991	I
IgG2b APC-Cy7	-	Rat	BD 552773	II
IgG2b BV510	-	Rat	Biolegend 400646	II
IgG2b FITC	-	Rat	AbD Serotec MCA1125F	I
IgG2c	-	Rat	Biolegend 400705	I
Langerin AF647	Mouse	Rat IgG2a	Dendritics DDX0362A647-100	II
Ly6C	Mouse	Rat IgG2c	Biolegend 128006	I
Ly6G	Mouse	Rat IgG2a	BD 551461	I
MHC II BV510	Mouse	Rat IgG2b	Biolegend 107635	II
normal rabbit IgG	-	Rabbit	Santa Cruz sc-3888	II
Podoplanin APC	Mouse	Hamster IgG	Biolegend 127410	II
Podoplanin PE	Human	Rat IgG2a	Biolegend 337004	II
Relm alpha	Mouse	Rabbit	Abcam ab39626	I
Ym1	Mouse	Rabbit	Stemcell 01404	I

4.2 Primers and probes

Table 2. Summary of all primers and probes used in the studies.

Primer/Probe	Target Species	Product #	Used in
ACTB	Human	Thermo Fisher Hs99999903_m1	II
Actb	Mouse	Thermo Fisher Mm00607939_s1	I
ADORA1	Human	Thermo Fisher Hs00181231_m1	II
ADORA2A	Human	Thermo Fisher Hs00169123_m1	II
ADORA2B	Human	Thermo Fisher Hs00386497_m1	II
ADORA3	Human	Thermo Fisher Hs00181232_m1	II
ANGPT2	Human	Thermo Fisher Hs00169867_m1	II
Arg1	Mouse	Thermo Fisher Mm00475988_m1	I
B2M	Human	Thermo Fisher Hs99999907_m1	II
BST2	Human	Thermo Fisher Hs00171636_m1	II
CCL19	Human	Thermo Fisher Hs00171149_m1	I
CD69	Human	Thermo Fisher Hs00934033_m1	II
CLEVER (UPL-probe #74)	Human	left: cac atg tgc caa gaa gat cc right: cac agc gtg cca aag aaa c	II
ELOVL6	Human	Thermo Fisher Hs00907564_m1	II
EPSTI1	Human	Thermo Fisher Hs01566789_m1	II
ERG	Human	Thermo Fisher Hs05021992_s1	II
HES1	Human	Thermo Fisher Hs00172878_m1	II
ICAM1	Human	Thermo Fisher Hs00164932_m1	II
IFI6	Human	Thermo Fisher Hs00242571_m1	II
IGF1	Human	Thermo Fisher Hs01547656_m1	II
IL6R	Human	Thermo Fisher Hs01075666_m1	II
KDR	Human	Thermo Fisher Hs00911700_m1	II
Mrc1	Mouse	Thermo Fisher Mm00485148_m1	I
MX1	Human	Thermo Fisher Hs00895608_m1	II
Nos2	Mouse	Thermo Fisher Mm00440485_m1	I
NT5E	Human	Thermo Fisher Hs00159686_m1	II
Nt5e	Mouse	Thermo Fisher Mm00501915_m1	I
OAS2	Human	Thermo Fisher Hs00942643_m1	II

4.3   Methods

Table 3.   Summary of the methods used in the studies. Brackets indicate methods that were performed in the study but were not included in the publications.

Method	Used in
Adherence assay	II
Cell isolation and culture	I, II
Cell sorting	II
CRISPR/Cas9	II
Cytokine measurements	I
Dot Blot	[II]
Enzymatic assays	I, II
Flow cytometry	I, II
In vivo: FITC ear painting	II
In vivo: footpad injections	II
In vivo: Oxazolone model	II
In vivo: peritoneal injections	I
RNA isolation, cDNA synthesis and qPCR	I, II
RNA sequencing	II
Single cell sequencing	II
siRNA silencing	II
T-cell proliferation	[II]

4.3.1   Adherence assay (II)

Human dermal lymphatic endothelial cells (HDLECs, PromoCell, Heidelberg, Germany) were silenced with siRNA specific for CD73 or a control construct (Dharmacon, Lafayette, USA) and plated on µ-slides VI from IBIDI (Martinsried, Germany). The next day, 5000 carboxyfluorescein succinimidyl ester (CFSE)-labelled immature or mature moDCs were added to the confluent cultures, let adhere for 45 minutes and were fixed with paraformaldehyde after non-adherent cells were removed. The slides were recorded and the number of adherent cells determined with ImageJ. Similarly, this was also done for control-siRNA-treated HDLECs.

4.3.2   Cell sorting (II)

Human dermal microvascular endothelial cells (HDMECs) from juvenile foreskin obtained from PromoCell (Heidelberg, Germany) were labelled with anti-Podoplanin-PE and sorted on a BD FACSAria with the 100µM nozzle into Podoplanin-positive and Podoplanin-negative cells.

4.3.3 Dot Blot ([II])

To verify that treatment of the endothelial cells with the 4G4 antibody cells leads to a shedding of CD73, we performed dot blot experiments. HDMEC cells (PromoCell, Heidelberg, Germany) were blocked with 10 µg/mL of three different CD73 antibodies (118, AD2 and 4G4) for 2 days before supernatant and cells were collected. Cells were then lysed in lysis buffer for 30 minutes on ice before transferred onto a presoaked nitrocellulose membrane. As a lysis buffer PBS with 50 mM β-octyl glucoside, 1mM PMSF and 1% Aprotinin was used (Sigma, Helsinki, Finland). The membrane was dried, washed with PBS/Tween and blocked over night with the Odyssey blocking buffer (Licor, Bad Homburg, Germany). This was followed by another wash, 10 minutes of blocking and staining with an anti-mouse secondary antibody conjugated with IRDye 800CW (Licor, Bad Homburg, Germany). The membrane was then recorded with an Odyssey CLx imaging system (Licor, Bad Homburg, Germany).

4.3.4 Enzymatic assays (I, II)

Enzymatic activities of different cell types and following different treatments were determined by using a method developed by Dr. Gennady Yegutkin. Here, tritium-labelled AMP, ADP or ATP were used as tracer substrates and the transfer of their radioactive phosphate was determined by scintillation counting. A detailed description can be found in studies (I) and (II).

4.3.5 Flow cytometry (I, II)

Single cell suspensions were incubated with human Ig (in the case of human cells) or Fc block (in the case of mouse cells) to block non-specific binding sites. For surface stainings, the cells were incubated with 10µg/mL of antibody solution for 30 minutes on ice and in the dark. The same staining procedure was repeated in case a conjugated secondary antibody was required. For staining intracellular epitopes, the cells were permeabilized with Perm/Fix from BD according to the manufacturer's instructions before staining the cells with antibodies. After staining, the cells were washed and recorded on a BD LSR II or BD Fortessa flow cytometer. In some cases, samples were fixed after the staining process with a 4% paraformaldehyde (PFA) solution.

4.3.6 *In vivo* experiments (I, II)

All *in vivo* experiments were performed with pathogen free mice of a C57BL/6J background that had originally been obtained from The Jackson Laboratory and

were bred in-house. The animals were two to three months of age and were either deficient for CD73 (CD73$^{-/-}$) or of a wildtype genotype. Animal experiments were done in compliance with the 3R principle and approved by the Finnish Animal Ethics Committee.

4.3.6.1   Footpad injections (II)

Footpad injections were performed by injecting 25 µL of a mixture of 50 µg ovalbumin diluted 1:1 in PBS and incomplete Freund's Adjuvant into the footpad of CD73 deficient and WT mice.

Following the injections, the animals were sacrificed one day later and their lymphnodes collected.

4.3.6.2   FITC ear paintings (II)

The dorsal sides of the mouse ears were coated with an irritant FITC solution where FITC was dissolved in a 1:1 solution of Acetone and Dibutylphtalate. Two days later DCs from draining lymph nodes were collected and analyzed by flow-cytometry. Detailed methods can be found in study (II).

4.3.6.3   Oxazolone model (II)

To induce skin inflammation, animals were sensitized by applying a 2% Oxazolone solution to their skin and challenged 5 days later with a 1% Oxazolone solution applied to their ears and skin. Skin thickness was measured and the skin collected and used for tissue stainings.

4.3.6.4   Peritoneal injections (I)

Peritoneal macrophages were polarized by injecting 800µL of a 4% thioglycollate solution together with either 5 µg LPS or 5 µg IL-4 and 25 µg of an anti-IL-4 antibody (BD Biosciences, Vantaa, Finland) into the peritoneum of wildtype and CD73 deficient mice. The IL-4 injections were repeated on day 2 but this time they were diluted in 200 µL PBS. Polarized cells were then harvested 16 hours later (after LPS injection) or 4 days after the initial injections. Extracted cells were then labelled and analyzed with flow cytometry.

4.3.7 RNA isolation, cDNA synthesis and qPCR (I, II)

The NucleoSpin RNA II kit from Macherey Nagel was used for the extraction of cellular RNA. Where cDNA was required, the extracted RNA was reverse-transcribed with the iScript cDNA synthesis kit from BIO-RAD (in study (I)) or the SuperScript VILO cDNA Synthesis Kit from Thermo Fisher Scientific (in study II). With the generated cDNA different genes of interest were analysed either by using Applied Biosystems TaqMan Gene Expression assays (studies (I) and (II)) or by using the UPL-probe library (study (II)) from Roche. The used primers/probes can be found in the original publications and in table 3.

4.3.8 T-cell proliferation ([II])

T-cell proliferation assays were performed with cells obtained from buffy coats (Finnish Red Cross Blood Service, Helsinki) or fresh blood. To obtain monocyte derived DCs, monocytes were extracted by using the Classical Monocyte Isolation Kit (Miltenyi Biotec) and cultured as described in the original publication (II). T cells were either first cultured as whole PBMCs and stimulated with Tetanus Toxoid (TT) by culturing the cells for 5 days with 20 µg/mL TT before purifying the T cells, or extracted immediately by using Miltenyi's Pan T Cell Isolation Kit. In all culture conditions, CD73 KO/silenced and their respective control HDLECs were used. Before the co-cultures, T cells were labelled with FITC (2.5 µM, 12 min, 37˚C, Sigma) and proliferation was induced by either using Human T-Activator CD3/CD28 Dynabeads (Thermo Fisher Scientific) according to the manufacturer's instructions, a 1X concentration of a 500X Phytohemaglutinin-L (PHA-L) solution (00-4977-03, eBioscience) or coculture with matured moDCs (10:1 ratio) and TT (20 µg/mL). Cells were cultured in IMDM media (Thermo Fisher Scientific) supplemented with 10% FCS, 0.4 mM GlutaMax (Thermo Fisher Scientific), 50 U IL2/mL and 5 ng IL-7/mL (both from Peprotech). The proliferation of CD4$^+$ and CD8$^+$ cells was then determined with flow cytometry on day 5 by assessing the dilution of CFSE.
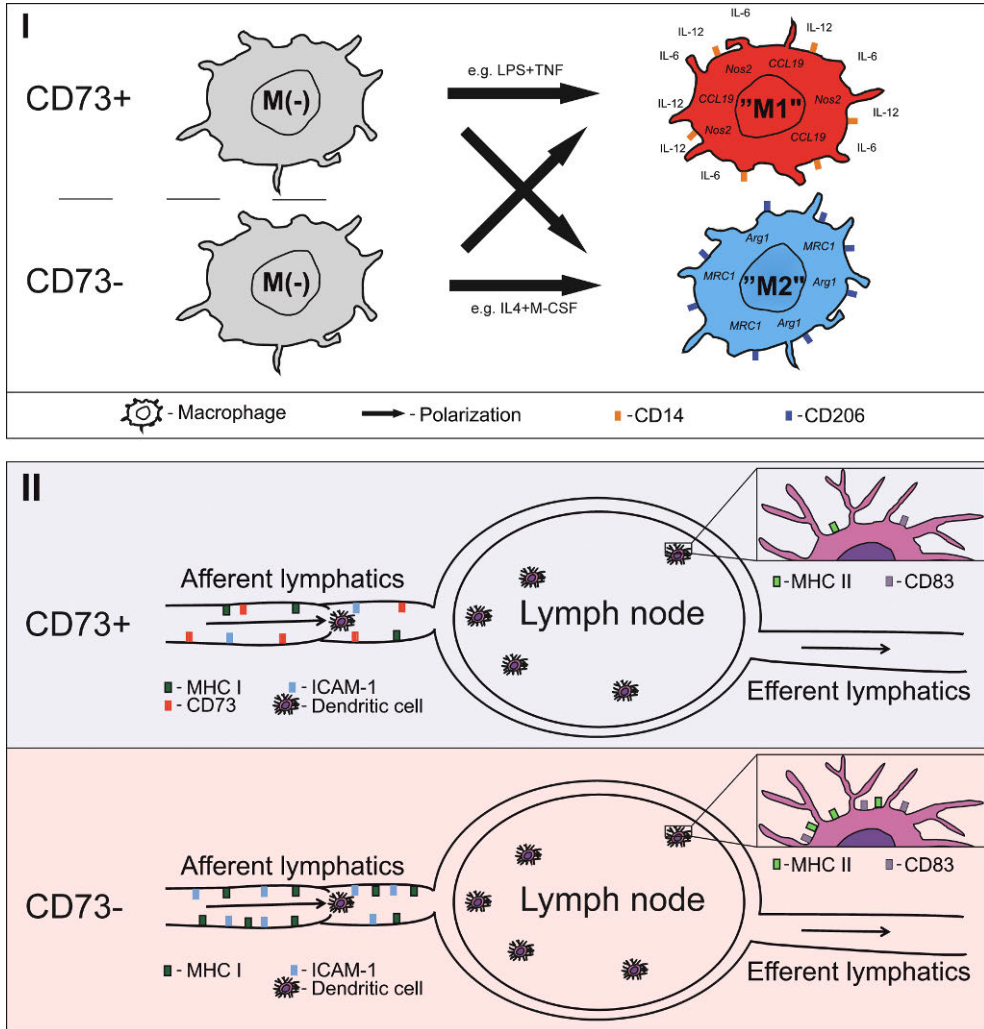
5   Results

Figure 5. Graphical summary of the main results from study I and II. **(I)** shows similar polarization of macrophages regardless of the presence of CD73. **(II)** shows that the absence of CD73 in afferent lymphatic vessels leads to an elevated expression of inflammatory molecules and stronger maturation of migrating DCs.

5.1 Human anti-inflammatory monocytes/ macrophages do not induce CD73 and their polarization is independent from the enzymatic activity of CD73 (I)

As CD73 has been shown to have anti-inflammatory properties in a multitude of tissues and conditions, we investigated its importance on one special type of immune cell: macrophages. As these cells can promote as well as resolve inflammations, we wanted to determine if CD73 is involved in these processes. To study this, we generated pro- and anti-inflammatory macrophages from MACS-beads purified human monocytes by exposing the cells to LPS and TNF or IL-4 and M-CSF, respectively. The polarization status was then verified by comparing the gene-, cytokine- and protein-expression of the cells. This confirmed their phenotype, as pro-inflammatory cells, i.e. cells that had been exposed to LPS and TNF (M(LPS+TNF)), showed a higher expression of known markers such as *CCL19*, IL-6 and IFN-γ, while on anti-inflammatory cells, i.e. cells exposed to IL-4 and M-CSF (M(IL-4+M-CSF)), the expression of *MRC1* and CD206 was increased (I, Fig. 1, Fig. 2 and S2 Fig.)

When we then determined protein- and gene-expression levels of CD73 on these cells, we unexpectedly could see that the originally CD73 negative monocytes had become positive only after being polarized to a pro-inflammatory direction, while anti-inflammatory cells stayed negative (I, Fig. 2). This finding was additionally supported by performing enzymatic assays that showed significantly higher AMPase (=CD73) activity in M(LPS+TNF) cells.

To investigate this further and to determine if the enzymatic activity of CD73 would affect the polarization of these cells, we used the CD73 inhibitor AMPCP or AMP, the substrate for CD73, in additional polarization experiments. Although the addition of AMPCP to the culture resulted in reduced protein levels of CD73 on pro-inflammatory M(LPS+TNF) cells, it did not affect mRNA levels and, more importantly, did not alter the polarization status of these monocytes/macrophages (I, Fig. 2). Similarly, AMPCP did not alter the phenotype of anti-inflammatory M(IL-4+M-CSF) cells and the addition of AMP did result in virtually identical cell polarizations in both the pro- and anti-inflammatory setting (I, Fig. 2 and S7 Fig.). These results therefore show that CD73 activity on monocytes/macrophages is not required for their successful polarization.

5.2 Murine peritoneal macrophages express CD73, polarize normally in its absence and are CD73-negative after polarization (I)

To investigate the importance of CD73 for macrophages further and in order to be able to study it in an *in vivo* setting, we utilized CD73-deficient and wild type mice.

Unlike the human cells we had studied, the peritoneal macrophages we obtained from wild-type mice expressed CD73 in their basal state before any intervention as determined by flow cytometry and qPCR (I, Fig. 3). Nevertheless, when we polarized the peritoneal cells towards a pro-inflammatory or an anti-inflammatory direction by injecting either LPS or a combination of IL-4 and an anti-IL-4 antibody (=IL4c), respectively, into the peritoneum, we found otherwise virtually identical phenotypes between wildtype and CD73-deficient animals. At the genomic level, the induction of *Nos2* in pro-inflammatory cells as well as *Mrc1* and *Arg1* in anti-inflammatory cells was comparable between the two genotypes. This was also reflected in the similar protein expression of different molecules such as Ym1, RELM-alpha or CD206 as well as similar cytokine secretion (IL-6, IL-12, KC (CXCL1)), whereas only a few cytokines such as G-CSF, IL-3, MCP-1 or RANTES showed slightly altered levels (I, Fig. 4 and S6 Fig.).

As not only the extent of polarization is important for the proper function of these cells but also their numbers, we additionally investigated if CD73-deficiency affected the cell numbers in the peritoneum. We therefore flushed the peritoneum of control-treated animals and animals that had been injected with LPS or IL4c and counted the extracted cells. Overall, the cell numbers were rather similar between the two genotypes following the different treatment modalities. However, there were elevated numbers of leukocytes detectable in KO animals following the polarization towards an anti-inflammatory phenotype. Nevertheless, the proportions of myeloid cells ($Ly6C^+/Ly6G^+$ or $CD11b^+$), granulocytes ($Ly6G^+$) and macrophages ($Ly6C^+$ or $F4/80^+$ or $CD206^+$) amongst these cells were comparable (I, Fig. 5).

Interestingly, when we investigated the expression of CD73 on elicited (or elicited and polarized) macrophages in the peritoneum, CD73 was absent from these cells. In contrast, CD73 expression on lymphocytes obtained from the peritoneum remained the same, whereas pro-inflammatory stimuli seemed to increase its expression on granulocytes (I, Fig. 3).

Overall, much like in the experiments with human cells, CD73 appeared not to have an essential role for the polarization of murine macrophages.

5.3 Silencing of CD73 on lymphatic endothelial cells causes a more pro-inflammatory phenotype (II)

Although it has been known that CD73 is expressed on the blood and lymphatic endothelium (Niemelä et al., 2008; Sleeman et al., 2001), most attention has been given to the study of blood endothelial cells (BECs), thereby neglecting lymphatic endothelial cells (LECs). This study had therefore been carried out in order to shed some light on the previously unknown role of CD73 in the afferent lymphatics.

To address this question, we used CD73-targeting siRNA silencing and the CRISPR/Cas9 knockout technique to reduce CD73 expression in human LECs that had been obtained from juvenile foreskins. With these cells we were able to reduce their CD73 expression on the genomic level on average by 90%, whereafter the gene expression profiles of these cells were then determined by applying RNA-sequencing and Single Cell Sequencing (II, Fig. 1). The results revealed significant alterations of more than 7500 genes and a plethora of changed pathways as determined from the KEGG and GO databases. Among the altered pathways were "purine metabolism"-, "cytokine activity"- or "inflammatory response"-pathways as well as a multitude of other inflammation-associated pathways (II, Fig. 1).

As CD73 is known to be intimately involved with the immune system and inflammatory processes in different cells and tissues, we were not surprised to find a predominance of upregulated inflammatory genes in our dataset, as well as in subsequent qPCR analyses. Among them were genes such as *CD69*, *TGFB1* and genes from the HLA-family (II, Fig. 2).

As lymphatic endothelial cells provide a transport system for different types of immune cells, we next investigated whether we could find changes in molecules that are known to interact with the cells of the immune system. As dendritic cells (DCs) are a particularly important cell type that can be found in the lymphatic system, we were excited to see that multiple genes on the LECs that coded for molecules interacting with DCs had been altered. Among these were genes for receptors (e.g. HLA A, HLA B, HLA C), adhesion molecules (e.g. ALCAM and ICAM-1) as well as genes for chemokines (e.g. IL-6, IL-7 or IL-33) (Table 4 and II, Fig. 2).

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Table 4. Silencing CD73 alters gene expression levels of endothelial receptors and chemokinespotentially interacting with dendritic cells

Category	Endothelial cell molecule	Fold change (log 2)	Target on dendritic cells
Immune receptors	MHC Class 1		
	HLA A	0.96	CD85a (LILRB3)
	HLA B	0.85	CD85c (LILRB5)
	HLA C	0.44	CD85d (LILRB2)
	MOCS1	-0.52	CD319 (SLAM7F)
	NCAM	1.13	CD171 (L1CAM)
	STIM1	0.27	CD300b (LMIR5)
Adhesion molecules	ALCAM	-0.4	CD6
	ICAM-1	1.4	CD18 (integrin b2)
			CD43 (sialophorin)
	MMP24	0.98	CD44
	ST6GAL1 (2,6 sialic acid)	0.38	CD22 (siglec-2)
Chemokines	CXCL11	1.84	CXCR3
	CXCR4	-0.4	CXCL12 (SDF-1)
	IL-6	-0.44	IL6R
	IL-7	-0.5	IL7R
	IL-32	0.98	IDO
	IL-33 (IL-1 family)	1.55	ST2

In addition to the alterations at the mRNA level, we could verify a similar significant increase at the protein level when we measured MHC class I and ICAM-1 expression by flow cytometry on CD73-silenced and Crispr/Cas9-treated cells (II, Fig. 3). Similarly, when comparing ICAM-1 levels on the lymphatic endothelium between CD73-deficient and wildtype mice we found a trend towards a higher expression in KO animals (II, Supporting Information Figure 11).

5.4 Lymphatic CD73 reduces the maturation of adjacent DCs *in vitro* and *in vivo* (II)

To determine if the changes we had observed on the LECs also resulted in an altered phenotype of DCs that interact with them, we investigated their interaction in coculture experiments in more detail. First, we wanted to follow-up on the changes of ICAM-1 and therefore tested whether DCs adhered differently to

CD73-deficient LECs. As Podgrabinska et al. (2009, Journal of Immunology) had reported that immature DCs not only bind more frequently than matured DC but also were much more sensitive to the presence of molecules such as ICAM-1, we started by using those cells. We could observe that more DCs bound to LECs that were either inflamed or CD73-deficient and thereby had elevated levels of ICAM-1 (II, Fig. 3). We then performed the same experiment with partly-matured DCs, as DCs of this phenotype are most commonly found migrating in the lymphatics. For this, we established a maturation protocol that yielded a solid upregulation of different DC maturation markers without maturing the DCs completely (Figure 6). However, in contrast to immature DCs, we could not observe an altered binding due to differences of CD73 levels with these cells (II, Fig. 3).
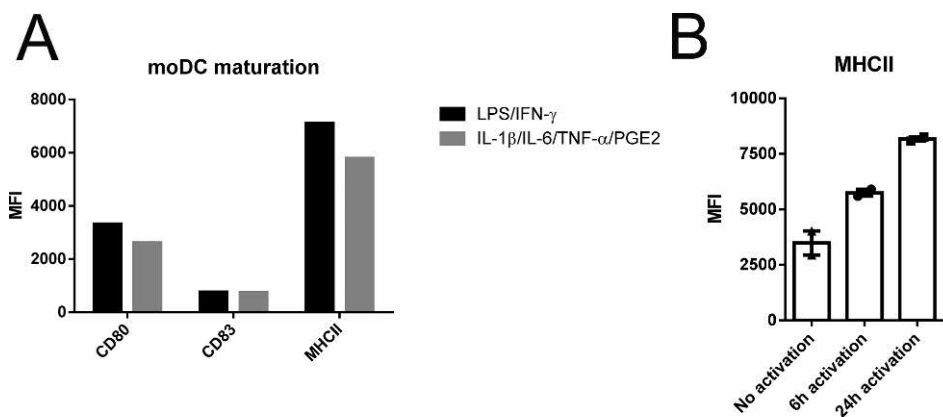
Figure 6. **(A)** Comparison of different maturation methods and their effect on established DC maturation markers after 24h. **(B)** MHCII levels of not matured moDCs and 6h or 24h after exposure to LPS and IFN-γ (n=1-2).

In addition to their binding capacity, we also investigated if the maturation of DCs was influenced by the presence of LECs and specifically by the expression of CD73 on co-cultured LECs. We therefore first co-cultured either non-activated or activated moDCs together with LECs and determined the expression of the maturation markers CD40, MHCII, CD80, CD83 and CD86 on the DCs (Figure 7). Overall, the maturation status following the co-culture was very similar to the culture of moDCs alone, with only a small reduction of CD83 levels being detectable on activated moDCs (Figure 7).
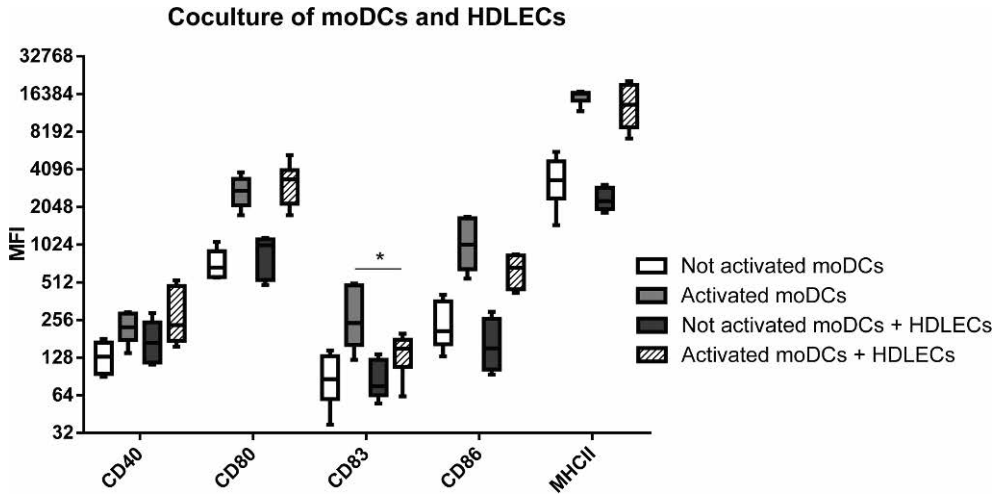
Figure 7. DCs co-cultured together with human dermal lymphatic endothelial cells (HDLECs) show very similar expression of their maturation markers compared to DCs cultured alone, except for a slightly reduced expression of CD83 as determined by flow cytometry (n=5-7).

To thoroughly study what effect the expression of LEC-CD73 has on co-cultured DCs, we used a pool of different siRNA constructs, as well as CRISPR/Cas9, to reduce the expression of CD73 on LECs before we cultured them together with maturing moDCs or primary skin-derived DCs and Langerhans cells. Overall, the results were quite similar as we detected upregulation of different DC maturation markers in all settings when endothelial CD73 had been reduced (II, Fig. 3). Most commonly, CD83 and MHCII were upregulated following the coculture. Interestingly, in cultures of siRNA-silenced LECs and moDCs or skin-derived DCs, CD80 was found to have a reduced expression. Furthermore, when CRISPR/Cas9 had been used for disrupting CD73 expression, also significant increases of CD40 and CD86 could be seen (II, Fig. 3).

These changes, together with the downregulation of the gene coding for the A2b adenosine receptor in moDCs, therefore demonstrated a more matured/inflammatory phenotype of DCs in the absence of CD73 (II, Fig. 3). Interestingly, the observed changes seem to be contact-dependent as neither culturing DCs with the culture supernatant from LECs nor interfering with the enzymatic cascade important for the dephosphorylation of AMP to adenosine were having a similar effect on the measured DC maturation markers (II, Fig. 4). To determine if this effect can also be seen on other immune cells and additionally, if the different maturation of the DCs affects other cells, we investigated the proliferation of T cells. In the first approach, $CD4^+$ or $CD8^+$ T cells were labelled with CFSE and cultured together with CD73-deficient and control LECs. The

proliferation of the T cells was induced by either PHA-L or by anti-CD3/CD28 stimulator beads. Although the differences did not reach significance, following the PHA-L stimulation a nonsignificant trend towards an increased cell division rate of T cells that had been cultured with CD73-deficient LECs could be observed (Figure 8).
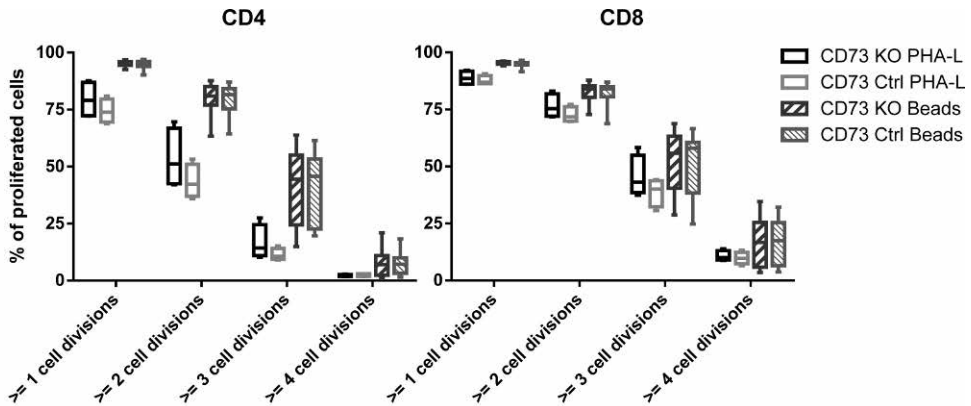

Figure 8. CFSE-labelled CD4 and CD8 T cells show a trend towards more proliferation when cultured with Crispr/Cas9-treated CD73-deficient LECs and stimulated with PHA-L. In contrast, no such trend could be observed when T cells were stimulated with CD3/CD28 Dynabeads (n=4-10, shown as boxplots).

However, in additional experiments using DCs and tetanus toxoid (TT) for stimulation the T cells did not show an altered proliferation (Figure 9).
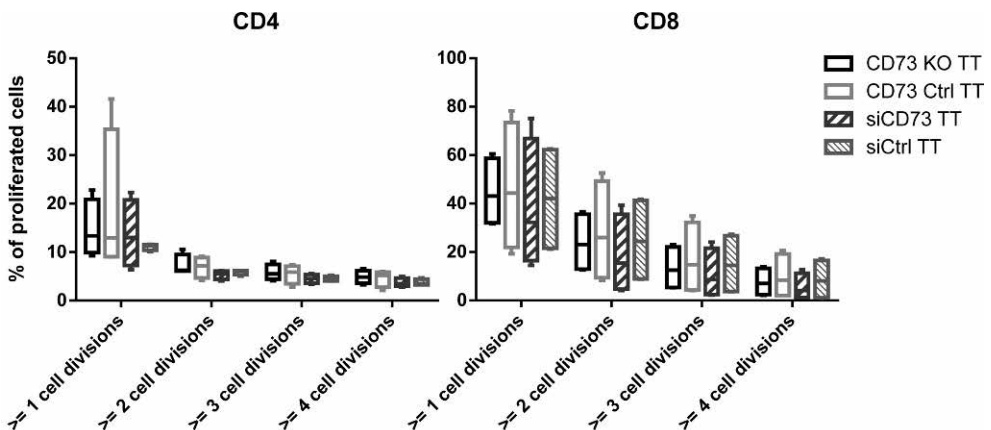

Figure 9. CFSE-labelled CD4 and CD8 T cells proliferated similarly when stimulated by DCs and TT and cultured together with CRISPR/Cas9, siRNA or control treated LECs (n=4, shown as boxplots).

Finally, as all our previous results were obtained from *in vitro* experiments, we decided to test whether the same effect can be seen when using an *in vivo* mouse model. To this end, we immunologically challenged CD73-deficient and WT mice either by injecting Ovalbumin and incomplete Freund's Adjuvant into the footpad or by applying an irritiant FITC-solution to the dorsal part of the ear. In both approaches, the dendritic cells from the draining lymph nodes were harvested and their maturation status was compared. Very similar to the results obtained from *in vitro* experiments, also *in vivo* the dendritic cells that had not been exposed to CD73 showed a more inflammatory phenotype as evidenced e.g. by increases of MHCII and CD40 (II, Fig. 5).

5.5 CD73 can be specifically blocked or reduced by antibodies with only minor effects on other genes or co-cultured DCs (II)

A different method to suppress CD73 besides intervention at the genomic level is to use antibodies. This approach is especially important as the use of antibodies in clinical trials is much more widespread compared to the use of siRNA or other genetic alterations. However, even though there are multiple clinical trials with antibodies ongoing (ClinicalTrials.gov. Bethesda (MD): National Library of Medicine (US). 2000 Feb 29 - ., 2015, 2016, 2017, 2018a, 2018d, 2018c, 2018b), their potential for side effects has not been evalutated in detail. We therefore used three different anti-CD73 antibodies that each interfered with CD73 in a specific way and determined their effect on LECs and BECs. We decided to include BECs in our analysis, as also BECs would be exposed to a therapeutically used anti-CD73 antibody and therefore might be affected.

All three antibodies (AD2, 4G4 and 118) specifically target human CD73 but differ in their blocking function. After binding to CD73, AD2 causes clustering and subsequent internalization of the molecule, while the binding of 4G4 triggers shedding of CD73. In contrast, 118 does not alter the physical presence of CD73 on the cell membrane (II, Fig. 6). We verified this by performing a dot blot assay both on the supernatant and on lysed cells after treatment with the different antibodies. While in all lysates CD73 molecules could be found, only following 4G4 antibody-treatment its presence could also be seen in the supernatant (Figure 10).
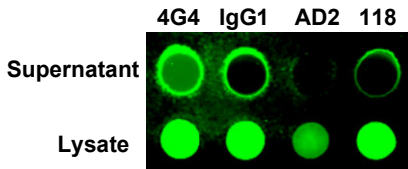
Figure 10. Dot blot assay showing the presence of CD73 molecules in the supernatant (top row) and lysed cells (bottom row) after treating LECs with 4G4, Control IgG1, AD2 and 118 antibodies (from left to right).

In addition, we evaluated the efficiency of the different methods (siRNA, antibodies and Crispr/Cas9) to reduce the CD73 protein expression. This revealed the highest efficiency (about 90%) with the genetical modifications, while using CD73-targeting antibodies reduced its expression by 60% to 80%. This is presented in Figure 11.
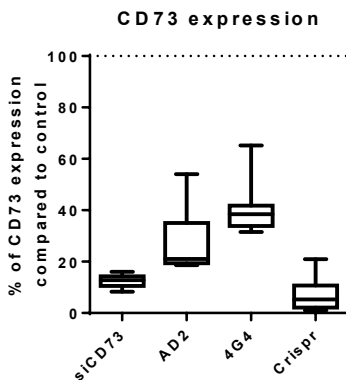

Figure 11. CD73 protein expression is reduced after treatment of LECs with CD73-targeting siRNAs, CRISPR/Cas9 or the antibodies AD2 and 4G4 (n=4-5). Data adapted from Eichin et al. 2020.

When looking at the enzymatic activity, the turnover rate of AMP had been reduced following the blocking with the different antibodies (II, Fig. 6). Interestingly, the extent of this reduction was very similar regardless of the used antibody and their specific effect on CD73 whereas the relative reduction was more efficient in BECs (>70%) compared to LECs (>50%). Furthermore, the activities of ATPase, ADPase and AK were unaltered by the antibodies in both endothelial cell types (II, Fig. 6).

As we wanted to analyze whether the use of antibodies would also affect CD73 expression at the genomic level and whether it had any further-reaching transcriptomic consequences, we performed RNA-sequencing of the endothelial cells. In contrast to the results from our siRNA silencing experiments, we only found a handful of genes that had been mildly affected (II, Fig. 7). Moreover, since in subsequent qPCR experiments none of them could be validated, the use of anti-CD73 antibodies does not seem to have a large effect on the transcriptome of endothelial cells.

Nevertheless, we were still curious whether antibody-mediated blocking of CD73 on LECs would have a similar effect on co-cultured DCs as we had seen with genetic interventions. We therefore performed similar experiments by culturing antibody-treated LECs and moDCs together and determined the maturation status of the DCs. However, regardless of the used antibody moDC maturation was not affected (II, Fig. 7). Overall, blocking CD73 on endothelial cells with antibodies does therefore not seem to affect interacting DCs and is likely negligible for this interaction.

6 Discussion

6.1 CD73 and macrophages – in men and mice (I)

Since its discovery, CD73 has been found to be expressed in numerous tissues and cells. The extent to which it is expressed varies, depending on the cell type as well as the physiological state that the cell is currently in. With its dephosphorylating enzymatic activity, CD73 is an essential part of the cascade that leads from pro-inflammatory ATP to anti-inflammatory adenosine, thereby converting AMP to adenosine. This quickly resulted in it being recognized as a key molecule able to regulate the extracellular purinergic signaling. With the well-established role of adenosine as an immune-regulating molecule, many research groups therefore began studying CD73 expression and function on different cells of the immune system, This newfound interest increased even more after it became clear that CD73 can be expressed not only on cells of the immune system but also on cancer cells.

However, as CD73 and its role on macrophages had not been studied very extensively, we decided to fill this void and studied the expression of CD73 on differently polarized human and mouse macrophages as well as its importance for their polarization (Eichin et al., 2015). We therefore applied different polarization protocols to monocytes/macrophages and could confirm the pro- or anti-inflammatory phenotype by looking at established markers. An inflammatory phenotype was for example indicated by an elevated expression of the gene CCL19 or the cytokines IL-6 and IL-12, while in an anti-inflammatory phenotype more CD206 protein could be detected (Belosevic et al., 1990; Biswas and Mantovani, 2010; Davies et al., 2013; Geissmann et al., 2010; Gordon and Martinez, 2010; Lawrence and Natoli, 2011; Liu and Yang, 2013; Martinez et al., 2014; Mosser and Edwards, 2008; Shalhoub et al., 2011; Sica and Mantovani, 2012; Sieweke and Allen, 2013; Tomioka et al., 2012; Wynn et al., 2013; Xue et al., 2014).

These successful polarizations lead us to the discovery that human monocyte derived macrophages can indeed express CD73, however only after being polarized towards a pro-inflammatory direction. In contrast, polarized mouse peritoneal macrophages were negative for CD73. In addition, in both humans and mice macrophage polarization was completely independent of CD73.

These findings are intriguing, as other immune cells such as e.g. Tregs require CD73-derived adenosine for their proper function (Deaglio et al., 2007; Junger, 2011; Linden and Cekic, 2012; Liu et al., 2011; Tiemessen et al., 2007) and also the anti-inflammatory phenotype of *in vitro* cultured peritoneal macrophages seems to be affected by CD73 (Csoka et al., 2012; Lopez-Castejón et al., 2011). In addition, in some mouse models CD73 had been associated with more anti-inflammatory cells (Lopez-Castejón et al., 2011; Zanin et al., 2012). Nevertheless, our finding of CD73 expression on human pro-inflammatory monocytes/macrophages, surprising at first, is in line with earlier reports that have shown CD73 expression on a sub-group of monocytes that are considered pro-inflammatory (Sciaraffia et al., 2014; Ziegler-Heitbrock, 2007). This expression, however, appeared not to be important for the polarization of the cells as blocking CD73 with AMPCP had no effect on it. As adenosine itself has the potential to affect macrophage polarization, this effect is therefore likely CD73 independent (Haskó and Cronstein, 2013; Haskó and Pacher, 2012; Németh et al., 2011).

However, it seems that the specific effect of CD73 is very much dependent on the used study system (i.e. cell type, species, *in vivo* vs. *in vitro*). While we could show that murine peritoneal macrophages do not upregulate CD73 following their polarization, a different experimental setup used in earlier studies came to a deviating conclusion (Lopez-Castejón et al., 2011; Mantovani et al., 2004; Zanin et al., 2012). This was likely due to the different origin of the macrophages, as in particular the population of peritoneal macrophages seems to change rather dramatically upon certain stimuli such as inflammation. This switches the local population from mainly large peritoneal macrophages (LPM) towards a majority of small peritoneal macrophages (SPM) (Cassado et al., 2015). These cells are not only of different origin as LPMs are of embryonic origin while SPMs are from bone-marrow-derived myeloid precursors, but also differ in the molecules that they express. LPMs express CD73 while SPMs do not, making it clear that when results from peritoneal macrophages are compared, their starting population has to be considered. In our *in vivo* experiments the main population of macrophages were CD73-negative SPMs, while experiments that showed CD73 expression after *in vitro* polarization of peritoneal macrophages had used CD73-positive LPMs (Lopez-Castejón et al., 2011; Zanin et al., 2012). Under specific circumstances, such as severe sepsis, the findings may however differ as it has been reported that without CD73 a more pro-inflammatory cytokine profile might prevail (Hasko et al., 2011).

Nevertheless, as macrophages from wildtype and KO animals showed basically identical polarization in our experiments and the small differences in secreted cytokines are possibly due to varying cell amounts, our data overall show that CD73 is dispensable for macrophage polarization in humans and in mice.

6.2 CD73, endothelial cells and dendritic cells (II)

Endothelial cells are distributed throughout the mammalian body and fulfill a multitude of different tasks. To do so efficiently, they have evolved into different subtypes with specific roles. When endothelial cells cluster together and form a single layer, this layer is called endothelium of which there are two different types, the blood endothelium and the lymphatic endothelium. Although both types are important in the control and transport of different cells and nutrients, they differ in their function, in what they transport, as well as in their composition and molecules expressed. Nevertheless, it has been shown that CD73 can be expressed on both types of endothelium, sparking interest in its tissue specific role. While blood endothelial cells have been studied in more detail, the role of CD73 on lymphatic endothelial cells has remained enigmatic (Ålgars et al., 2011; Takedachi et al., 2008). We therefore performed this study to remedy this.

In contrast to BECs, the expression of CD73 on LECs is not as constant and additionally differs significantly between different species (Yegutkin et al., 2015). In humans, efferent lymphatics are devoid of CD73 while afferent lymphatics are expressing high levels throughout the vessels (Ålgars et al., 2011). In contrast, afferent lymphatics in mice have a lower and more sporadic expression pattern of CD73 (Ålgars et al., 2011).

To get a better understanding of what role CD73 plays on the afferent lymphatics, we therefore performed siRNA and CRISPR/Cas9-silencing of HDMECs and sequenced their RNA. After carefully evaluating the results, we could attribute immuno-dampening effects to CD73 as its removal resulted in an increase of inflammation-associated genes, pathways as well as proteins. In addition to increases in inflammatory genes such as *CD69* and *TGFB1*, we also found elevated expressions of surface molecules such as MHCI and ICAM-1, both molecules that have been reported to be increased during inflammation (Frank and Lisanti, 2008; Mai et al., 2013). As ICAM-1 is an adherence molecule, we could confirm an increased adherence of immature DCs to CD73-silenced LECs, a finding in line with published data that showed the effect of elevated ICAM-1 levels on DC-binding (Podgrabinska et al., 2009). Interestingly, this change in the binding rate is much more pronounced in immature DCs compared to partly-matured DCs (Podgrabinska et al., 2009).

The overall pattern of our results was not that surprising, as it is well known that CD73 can exert anti-inflammatory functions, for example via adenosine (Challier et al., 2013; Linden and Cekic, 2012; Sidibé and Imhof, 2015). However, it was intriguing to see that silencing CD73 on LECs changed the interaction of DCs and LECs. It has been shown earlier, that the interaction of LECs and DCs leads to a reduced maturation and diminished inflammatory phenotype of the DCs (Butler et al., 2006; Podgrabinska et al., 2009). Our findings now suggest that

CD73 plays a part in this interaction. We found significantly higher expression of different DC maturation markers (MHCII, CD40, CD83, CD86), as well as reduced levels of the adenosine A2b receptor on DCs following coculture with CD73-silenced LECs, all consistent with a more pro-inflammatory profile (Banchereau and Steinman, 1998; Podgrabinska et al., 2009; Wilson et al., 2009; Zhou and Tedder, 2002). Although the exact results varied somewhat when different silencing/KO techniques or DCs from different sources were used, this variation is to be expected as different techniques always vary in their efficiency and specificity. In addition, the different source of the used DCs (moDCs vs. primary skin-DCs) and their status, together with the necessary steps for their extraction, purification, handling, culture and maturation are bound to cause some differences (Boltjes and van Wijk, 2014; Romani et al., 1989). Furthermore, our results additionally indicate that especially the direct interactions between DCs and LECs play an important role, while soluble mediator-mediated effects seem to be negligible. This is interesting, as for example adenosine has been shown to have immuno-regulating properties. However, these effects might be dependent on the studied cells and additionally on the local concentratins of cytokines, as it is known that for example adenosine has a rather short half-life (Löffler et al., 2007).

Futhermore, the fact that we obtained similar results from our *in vivo* mice experiments significantly strenghtens our data. Nevertheless, it has to be kept in mind that CD73 expression in mice differs from humans and that we compared wildtype animals to full CD73 KO mice and not lymphatic-tissue specific KOs. CD73 expression might therefore play a somewhat different role in the murine lymphatic sytem and our obtained results might additionally have been influenced by the absence of CD73 on non-lymphatic tissues and cells.

In addition to using genetical alterations, we also used CD73-targeting antibodies to study potential consequences of blocking CD73. In order to get an indication whether the clinical use of CD73-targeting antibodies has potential side effects affecting BECs or LECs due to changes to their transcriptome, we had blocked CD73 with three different antibodies. All antibodies blocked the enzymatic activity and the AD2 and 4G4 antibodies additionally changed the CD73 expression on the cell surface. However, none of them caused significant transcriptomic alterations (Airas et al., 1997b; Terp et al., 2013). This was the case for LECs as well as BECs. As the used antibodies had a similar efficiency as using siRNA in inhibiting the enzymatic activity but were less efficient to remove surface protein, it is possible that this reduction was not sufficient to induce a responsive genetical program that would affect CD73 transcription as well as the expression of other genes. It is also possible, that only through genetically modulating CD73 other genes are affected and alterations on the protein level are not efficiently transferred to the genomic level. These limited changes likely also explain some of

our other results, as they could explain why DCs had a similar phenotype when cultured with antibody-blocked LECs or cultured with control-antibody treated LECs.

It therefore seems that blocking CD73 with targeting antibodies on BECs or LECs, regardless of their function, is likely to pose only a small risk of adverse effects to these cells; good news when considering ongoing clinical trials that implement anti-CD73 antibodies (listed in the supplement of publication II).

Nevertheless, it is important to keep in mind that any intervention always has the potential for side effects. This is especially true when the intervention causes transcriptional changes. Although we tried to limit this possibility in our study by using different silencing approaches to alter CD73, it is possible that non-specific effects could have influenced the outcome.

Taken together, we could show in this study that CD73 expression on (afferent) LECs has an immuno-dampening role that limits or postpones a more inflammatory phenotype of LECs as well as of DCs that interact with LECs. This might therefore delay the maturation of DCs so that they only become fully matured once they reach the lymph node, ensures an optimal immune reaction. As this effect is only visible when genetically silencing CD73, it indicates a possible threshold rate of CD73 expression to function efficiently. Blocking CD73 with specific antibodies did not induce this effect, indicating that the used antibodies do not seem to alter CD73 sufficiently and therefore potential side effects of using them in clinical treatments are likely to be limited.

6.3 Implications and future perspectives (I–II)

The results we obtained in both our studies broaden the knowledge on the impact of CD73 in different aspects of the immune system. We showed that the effect and importance of CD73 is cell-type specific as its modulation on some cells changed their phenotype while on other cell types it did not (Figure 5).

As we found no impact of CD73 on the polarization of certain human and mouse macrophage subsets, it is tempting to conclude that macrophage polarization as such is completely independent of CD73. This could therefore have implications for clinical treatments. Considering that interfering with CD73 has become a popular potential treatment option for diseases such as cancer, acute respiratory distress syndrom or multiple sclerosis, these results largely rule out macrophages as a potential source of treatment effects.

However, as some macrophages are able to express CD73, this interpretation has to be approached with caution. It is possible that due to the heterogenity of macrophages, some specific CD73-dependent subtype exists. It would therefore be essential, that macrophages from other sources would be investigated and the

importance of CD73 for their function would be determined in order to understand the reason for CD73 expression. It would then be interesting to decipher if this expression has more than immune-regulating functions. As our work is one of only a few that did not only look at the expression of CD73 on different macrophage subtypes, but additionally investigated if altering CD73 affects their polarization capabilities, addressing this question in broader studies would certainly yield important results.

In contrast to our results with macrophages, the modulation of CD73 on LECs had a considerable impact on those cells. While we have focused on immunological aspects of these alterations, our RNA-seq dataset contains a plethora of unexplored gene alterations and therefore provides a good starting point, for us and other groups, to study CD73 also in other contexts.

Furthermore, our results from experiments using different CD73-targeting antibodies have several implications. The most obvious one was already discussed earlier, namely the fact that due to the limited side effects on BECs and LECs the use of those antibodies seems to be safe and therefore can be proceeded with in clinical applications as long as negative effects on other tissues can also be ruled out. In addition, it might be worth to investigate the exact mechanism why some effects, such as the dampening of the dendritic cells, could only be seen with genetic interventions and not be replicated by using antibodies. This would not only help to get a better understanding of the function of the antibodies and their intervention with CD73, but would also facilitate the development of new therapeutic antibodies. New antibodies could then be developed that would show a thorough removal of CD73 and therefore might improve the generation of a favorable, more pro-inflammatory, phenotype in certain disease conditions.

7 Conclusions

The immune system is a powerful tool that is exceptionally well adapted to deal with countless challenges. It can take care of various pathogens such as fungi, bacteria and viruses as well as of cells with aberrant growth. To do so, it is finely tuned and regulated and can switch from an activating state, in which it recruits, targets and destroys cells, to a deactivating state where it dampens and resolves inflammation and repairs tissues.

As this does not only require the synergy of different cells, but also the regulation of countless genes, receptors and cytokines, it is important to decipher the role of each molecule involved.

One such molecule is CD73.

As one of the most important ectoenzymes to dephosphorylate AMP to adenosine, CD73 has raised a lot of research interest in recent years in areas such as biochemistry, immunology as well as cancer research. However, the more interest a topic receives, the more it becomes clear how much there is to learn.

In our studies, we therefore tackled and resolved a small part of this puzzle, namely to decipher the importance of CD73 for macrophages and their polarization (study I), to determine the role for its expression on lymphatic endothelial cells (LECs) and evaluate the consequences of its inhibition (study II).

We hereby could show that CD73 can be present on human as well as on mouse monocytes and macrophages under certain circumstances, but that this expression is dispensable for the polarization as well as for the phenotype of these cells. Cells from both human and murine origin were able to polarize normally regardless of whether CD73 was expressed, knocked-out or blocked on those cells.

We then continued to investigate CD73 and its role in different parts of the immune sytem by deciphering the effects of its absence on LECs. By using RNA-sequencing, *in vitro* assays with human cells as well as *in vivo* mouse models we were able to show that the expression of CD73 on LECs has immune-dampening effects on the LECs themselves as well as on dendritic cells that interact with them. As these effects were small but consistent, we therefore concluded that CD73 contributes to anti-inflammatory effects mediated by LECs. Furtheremore, we showed that CD73-targeting antibodies only mildly affect LECs or blood

endothelial cells (BECs) and therefore are likely safe to be used in clinical treatments without the risk of severe side effects due to CD73 modulation on LECs or BECs.

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