TRIGGERS FOR THE RELEASE OF EXTRACELLULAR VESICLES FROM DENDRITIC CELLS:

IMPLICATIONS FOR ANTIGEN PRESENTATION

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Triggers for the release of extracellular vesicles from dendritic cells: implications for antigen presentation

Stimulatie van de productie van extracellulaire membraanblaasjes door dendritische cellen: implicaties voor antigeenpresentatie

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de
Universiteit Utrecht
op gezag van de
rector magnificus, prof.dr. H.R.B.M. Kummeling,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op

dinsdag 19 november 2019 des middags te 12.45 uur

door

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geboren op 20 april 1990
te Groningen

Colofon

Triggers for the release of extracellular vesicles from dendritic cells: implications for antigen presentation, Marthe F.S. Lindenbergh

Cover design by Boukje Lindenbergh-Mulder
Layout and design by Anna Bleeker | persoonlijkproefschrift.nl.
Printing: Ridderprint BV | www.ridderprint.nl

Promotor:

Prof. dr. W. Stoorvogel

Copromotor:

Dr. M.L. Boes

Dit proefschrift werd (mede) mogelijk gemaakt met financiële steun van de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO).

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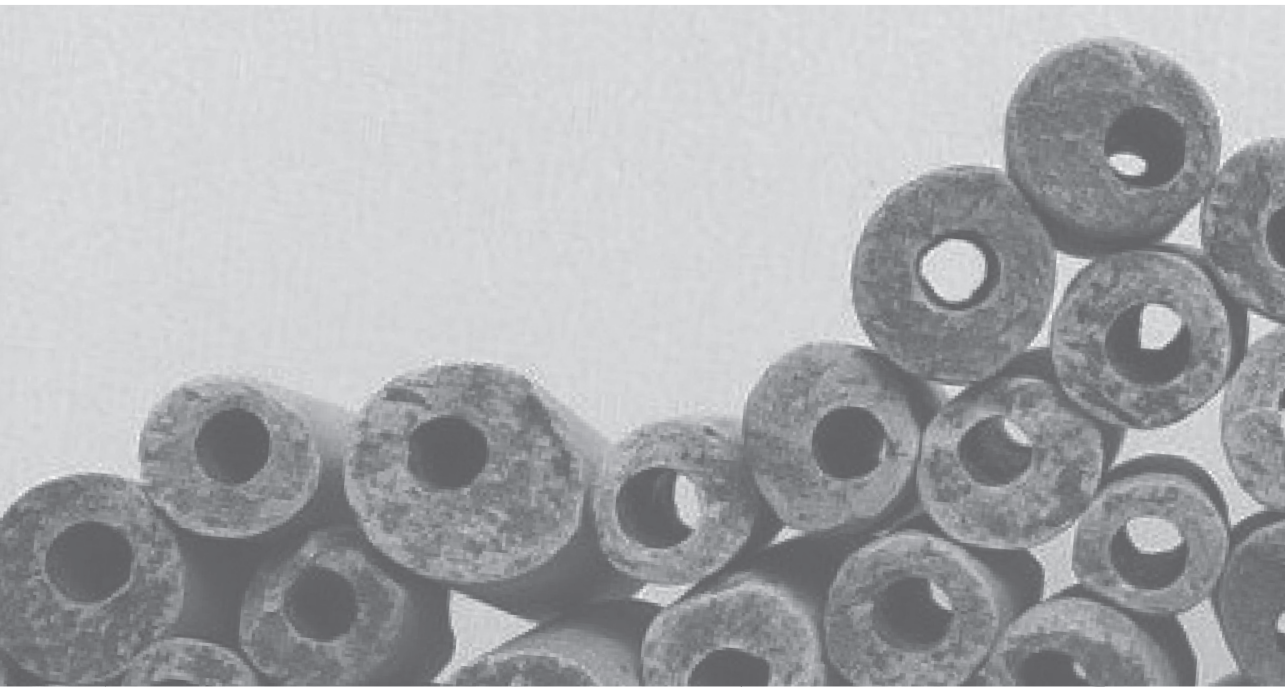

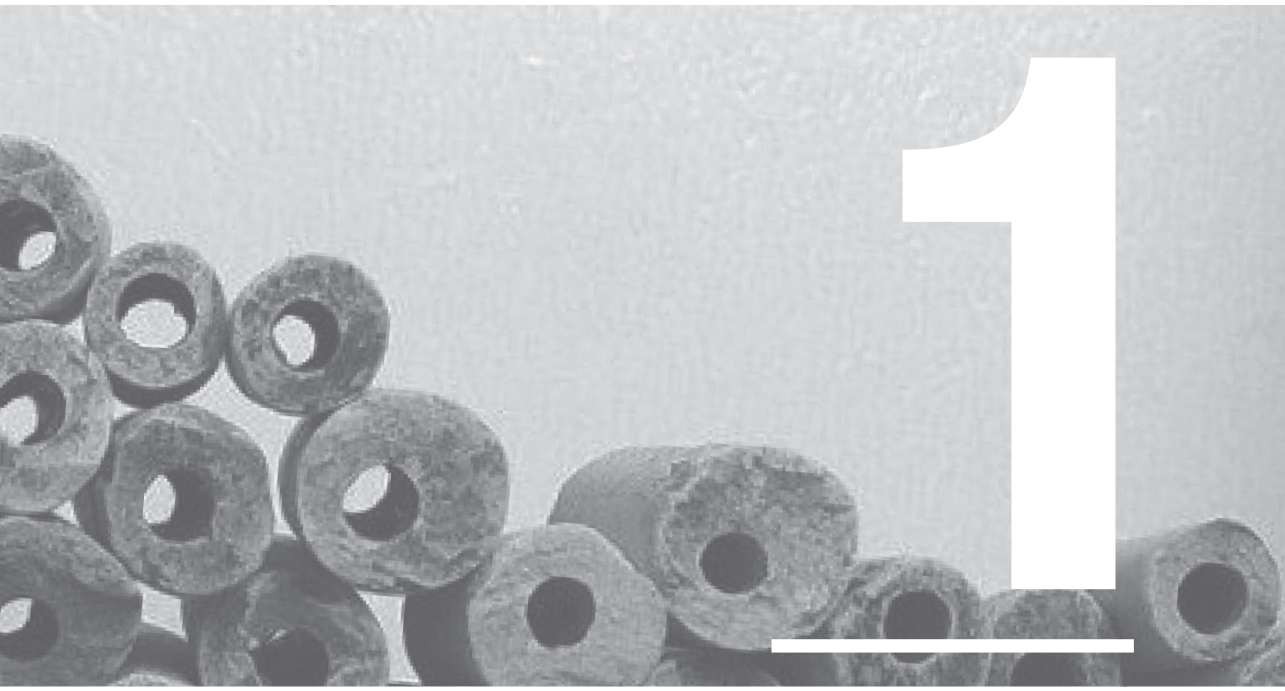

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General introduction

1. Dendritic cells and antigen presentation

Dendritic cells (DCs) are the sentinels of the immune system; they are present in almost every tissue and continuously sample their environment for danger signals. DCs take up extracellular material by employing both non-specific and receptor-mediated mechanisms of endocytosis, including receptor-driven clathrin-mediated endocytosis, micropinocytosis, macropinocytosis and phagocytosis[1,2]. As true sentinel cells, DCs express a wide variety of receptors that recognize pathogen-associated molecular patterns (PAMPs) or danger associated molecular patterns (DAMPs). Such pattern recognition receptors (PRRs) include Toll-like receptors (TLRs), C-type lectins, RIG-I receptors and NOD-like receptors, which upon receptor ligation can drive the maturation of DCs from sentinel cells into so-called antigen presenting cells (APC) and induce cytokine secretion[3]. In human DCs, up to 10 different TLR-subtypes are expressed either on the plasma membrane or intracellularly, albeit with different expression levels and downstream responses for distinct DC-subtypes[4] (see below). The various TLR subtypes bind molecular patterns that are more or less generically expressed by many pathogenic organisms: for example TLR4 binds lipopolysaccharide (LPS), a molecule derived from the outer membrane of gram negative bacteria, whereas TLR7 binds virus-derived single-stranded RNA. DCs are activated by ligation of their PRRs, resulting in the expression of immune modulatory receptors and co-stimulatory molecules at their plasma membrane, as well as in synthesis and secretion of immune regulatory cytokines.

DCs have a unique capacities to process endocytosed material into peptides that can be loaded onto either major histocompatibility complex (MHC) class I or MHC class II molecules. This endocytosed material may derive from pathogens, but can also originate from cancerous or virus transformed cells. Activated DCs migrate to lymphoid organs to present these MHC-peptide complexes to naïve T-cells, CD8+ T-cells and CD4+ T-cells for the initiation of adaptive immune responses (Figure 1). DCs can become activated directly by pathogens (**figure 1.1**). or alternatively, by immune-stimulatory cytokines that are released by other activated DCs in their vicinity (**figure 1.2**). DC-activation results in multiple phenotypic changes: the DCs develop membrane protrusions or dendrites[5] (**figure 1.3**)**,** and their phagocytic capacity is temporarily enhanced[6,7] (**figure 1.4**). This creates a window of opportunity to acquire a large pool of exogenous material to sample from for MHC-mediated peptide presentation[1,8,9]. To this end, DCs are stimulated to express and reroute peptide-loaded MHC-molecules to their plasma membrane. While undergoing phenotypical changes, activated DCs are also stimulated to migrate from the infection site through the lymphatics into the regional lymph node

to facilitate encounters with (cognate) T-cells[6] (**Figure 1.5**). To enable activation of (naïve) T-cells by DCs, expression of costimulatory molecules, such as CD80/CD86 and CD40 is increased. Adhesion molecules, such as ICAM-1, further enhance Interactions between DCs and T-cells[5] (**figure 1.6**).

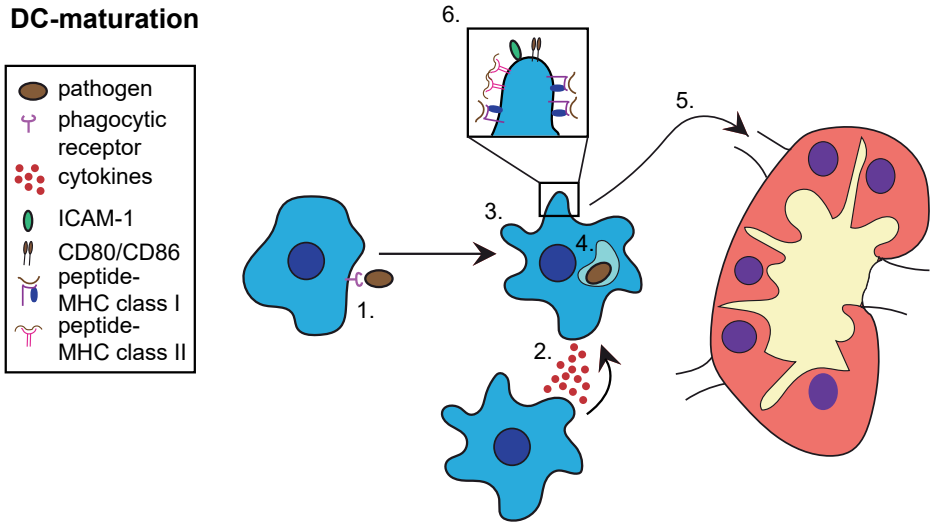

Figure 1: Maturation of DCs.
DCs can mature following 1. the uptake of pathogens or 2. Stimulation by cytokines derived from cells in their vicinity. This induces phenotypic changes, comprising: 3. the formation of dendrites, 4. an increase in phagocytotic as well as macropinocytotic uptake, 5. DCs also obtain the ability to travel towards lymph nodes, where they can interact with cells from the adaptive immune system. 6. Increased expression of antigen-presenting molecules and costimulatory molecules as well as other accessory molecules for interaction with T-cells enables DCs to activate (naïve) T-cells.

As described above, multiple phenotypic changes enable DCs to productively present antigens to (cognate) T-cells. Unique to DCs is their ability to cross-present exogenously acquired antigens on MHC class I, a phenomenon referred to as cross-presentation (see below: 1.2). DCs can activate T-cells harboring an antigen-specific T-cell receptor via presentation of specific antigenic peptides on MHC complexes. This is the first signal for T-cell activation[1,10]. T-cell activation is only accomplished when the DC additionally presents the appropriate costimulatory molecules[5] (signal 2) and secrete stimulatory cytokines[4] (signal 3).

1.1 DC-subsets

DCs can either be immunogenic or tolerogenic. At steady-state conditions, in the absence of DAMPs or PAMPs, DCs already display peptide-loaded MHC class I and II complexes at the cell surface. Such immature DCs present self-peptides, obtained as degradation products of endogenous proteins from the DC itself. Alternatively, the peptides can also derive from proteins from body fluids or apoptotic cells that were endocytosed/phagocytosed[11]. Presentation by immature DCs of such self-peptide-MHC complexes to T-cells that are reactive to self-derived peptides helps maintaining central tolerance by inducing inactivation (anergy) or elimination of self-antigen-specific T-cells[12] , or the development of regulatory T-cells (Treg)[13,14]. Activated immunogenic-peptide-presenting DCs on the other hand, instigate an immune response against invading pathogens. Together, tolerogenic and immature DCs maintain peripheral tolerance, thus preventing activated, immunogenic DCs from causing autoimmune responses. The outcome of a DC-T cell interaction therefore depends on the maturation status of the DC, for which the expression level of costimulatory molecules and cytokine production are important determinants[5,15]. In addition to their state of maturation, DCs are classified as plasmacytoid (pDCs), which are found in blood as well as in lymphoid tissue, and "classical/conventional" or myeloid DCs (cDCs/mDCs). The latter subset comprises both migratory and tissue-resident DCs[16,17]. As these two DC-subsets express different sets of PRRs and thus recognize different pathogens, mDCs and pDCs have specialized functions. pDCs are specialized in antiviral defense while mDCs recognize both viruses and bacteria[4,5,18,19]. Of note, the capacity to cross present, and the peptides that can be cross presented differ between pDC and mDC subsets[16]. Additionally, there are two less conventional DC types: skin-resident Langerhans cells (LCs) and at inflammatory conditions, a fourth subset of "inflammatory" DCs which differentiates from blood-derived monocyte progenitors[20–22]. Of note, this last subset can be generated *in vitro* (moDCs), but it remains unknown to what extent the *in vivo* generated cells resemble their *in vivo* counterparts[17].

1.2 Peptide-loading of MHC class I

MHC class I is expressed by nearly all cell types and is composed of a beta-2-microglobulin subunit and a transmembrane heavy chain that can bind a peptide for presentation to CD8+ T-cells. After synthesis, MHC class I complexes are retained in the endoplasmic reticulum (ER) until they have acquired a peptide in the peptide-binding groove of the heavy chain. Peptides destined for presentation on MHC class I can be generated in the cytosol by the proteasome, which degrades ubiquitinated proteins, followed by translocation to the ER by tapasin (TAP)[6]. Prior to loading on MHC class I, the peptides are further processed by peptidases, present in both the cytosol and

the ER[23]. After peptide-loading, the peptide-MHC class I traffics through the secretory pathway towards the plasma membrane, thus allowing inspection by CD8+ T cells of the presented peptide. Peptide-MHC I complexes are routinely recycled from the plasma membrane into the endosomal system, where they can be (re)loaded with peptides, and traffic back to the plasma membrane, or are targeted for degradation in lysosomes[24].

Unique to DCs is their capacity to present peptides from exogenously acquired proteins by MHC class I, in a process called cross-presentation[25–27]. However, as stated above, not all DC-subsets are avid cross-presenters. There are two main pathways that have been proposed to play a role in the peptide-loading of exogenously-obtained antigens for cross-presentation: the "classical" or "cytosolic" pathway and the "vacuolar" pathway. In the cytosolic pathway, exogenous proteins are acquired via endocytosis and transferred across the endosomal/lysosomal/phagosomal membrane into the cytosol, for processing into peptides by the proteasome. This is followed by transporter associated with antigen processing (TAP)-dependent transfer of the peptides into the ER for loading onto MHC class I[16,26,28]. Alternatively, thus generated cytosolic peptides may be translocated across the phagosomal membrane in either a TAP dependent[29] or TAP-independent[30] manner for (re)loading onto phagosome resident MHC class I. For TAP-dependent transfer directly into the endocytic/phagocytic pathway a set of ER resident proteins, including TAP, appears to be transferred from the ER in a Sec22b dependent manner[31]. Sec22b knock-out mice have severe deficiencies in their cross-presenting capacity, illustrating the importance of TAP-dependent phagosomal loading of MHC class I in this process[32]. In case of "vacuolar" cross-presentation, endocytosed antigens are degraded by lysosomal proteases within endocytic compartments, and the resulting peptides loaded directly on MHC class I complexes present in the endosome[16,26,33,34]. For cross-presentation of exogenously acquired peptides on MHC class I, in most cases it is essential that DCs are activated by PRR-ligation and signaling[16,17,25]. However, lymphoid organ-resident cDCs have been shown also to cross present without activation[35], thus enabling cross-tolerance[36]. Phagosomal TLR4 signaling, established by presence of LPS within the phagosome, has been shown to result in the recruitment of MHC class I molecules towards the phagosome from a Rab11a+ positive endosomal reservoir[37]. Alternatively, MHC class I molecules destined for cross-presentation can be acquired from the plasma membrane or directly from the secretory pathway[28,38]. The endosomal/lysosomal pathway may store endocytosed antigens for long periods of time by preventing full antigen degradation[39,40]. Protease activity is limited both by low concentrations of lysosomal proteases, limited acidification of the phagosomal lumen, and by preventing fusion of phagosomes with lysosomes[28,41,42]. The efficiency of cross-presentation also depends on the receptor responsible for internalization of the

antigen as the intracellular trafficking of the antigen into endosomal sub-compartments affects the efficiency of presentation[43,44]. Finally, a delay in phagolysosomal fusion as a result of TLR4 triggering results in increased cross presentation[45].

1.3 Processing of MHC class II

Whereas MHC class I is normally expressed by all nucleated cell types, MHC class II expression is limited to professional APCs, including DCs, macrophages and B-cells, but also by some epithelial cells after stimulation. MHC class II is usually loaded with peptides with an exogenous origin: they are derived from endocytosed proteins, macromolecular complexes, or entire pathogens.

MHC class II is synthetized as a oligomeric complex consisting of an α-chain, β-chain, and invariant chain (Ii) that is targeted to endosomes and lysosomes for antigen loading[46]. In endosomes, Ii is degraded by proteases, enabling loading of peptides that are generated by proteolysis from endocytosed material[9,28]. Peptide loading can occur in maturing endosomes, as well as in phagolysosomes. Upon phagocytosis of extracellular particles, newly formed phagosomes mature through fusion with other compartments within the endocytic pathway and also acquire endosome-specific TLRs[47]. Ultimately, phagosomal cargo is degraded by lysosomal hydrolases in a mildly acidic milieu, resulting in the liberation of antigenic peptides that can be presented on MHC class II[1]. Of note, autophagy enables MHC class II to also present endogenous antigens, enabling presentation of virus-derived peptides by infected DCs[26,48]. MHC class II has a short-half life in immature DCs as a consequence of rapid sorting in endosomes for lysosomal targeting and degradation[49]. Upon activation of DCs, synthesis of MHC class II is temporarily increased, peaking at 2-4 hours post activation[50,51], while ubiquitination-driven sorting of MHC class II to lysosomes is abrogated[49]. Together, these phenomena result in a dramatic increase in the capacity of MHC class II antigen presentation by DCs[46].

The efficiency with which pathogen-derived peptides are presented on MHC class II is dependent on the presence of TLR-ligands in the phagosome[52]. Of note, PRR-signaling from a phagosome stimulates the efficiency of antigen presentation from within that specific phagosome, to a much greater extent than from other MHC class II containing endosomes/lysosomes within the same cell[52]. This mechanism may skew presentation of peptides from phagocytosed pathogens relative to self-peptides by the same activated DC[53].

Phagocytosis of pathogens can be induced via opsonization of the pathogen by IgG, which is bound and internalized by Fcγ-receptors (FcRs), resulting in activation of the. The presence of opsonized pathogens stimulates phagosome maturation, and DC-mediated CD4+ T-cell activation is stimulated by the concomitant presence of opsonizing IgG and pathogen within the same phagosome[54]. This is consistent with the idea that MHC class II molecules from these compartments are efficiently loaded with peptides and transferred for stable expression at the plasma membrane, thus stimulating non-self-presentation.

1.4 DC-driven T-cell activation

DCs play a crucial role in the activation antigen-specific naive CD4+ T-cells by presentation of cognate peptides on MHC class II, and CD8+ T-cells via presentation of cognate peptides in complex with MHC class I. However, activation of naive CD8+ T-cells by antigen-presenting DCs requires prior licensing of these DCs by CD4+ T helper-cells[55,56]. Only DCs that are first licensed by CD4+ helper T-cells can prime CD8+ T-cells to become capable of autonomous secondary expansion following re-encounter with antigen, resulting in immune memory[56]. In one model, CD4+ T-cell help is dependent on a productive interaction of CD40 on the DC with CD40 ligand on the activated CD4+ T-cell[57]. In another model, which is supported by *in vivo* evidence, CD4+ T-cells can acquire APC-like properties by recruiting peptide-MHC class I (and co-stimulatory factors) from DCs in response to cognate peptide-MHC class II- T-cell receptor interactions[58–62]. This requires intercellular transfer of membrane proteins, a process that can me mediated by exosomes. (described below). Thus, CD4+ T-cells may acquire an APC-like phenotype by recruiting DC-derived exosomes, and these APC-CD4+ T-cells can then potentially activate CD8+ T-cells during subsequent interactions.

For effective CD4+ and CD8+ T-cell activation, it is important that both MHC class I and class II complexes on activated DCs are sufficiently loaded with pathogen/malignant cell specific peptides. As indicated above, maturing DCs increase their expression of MHC complexes at the plasma membrane. However, only a minority of these MHC molecules truly displays peptides from endocytosed/phagocytosed material[1]. Indeed, MHC class I complexes presenting peptides from endogenous proteins greatly outnumber those presenting exogenous antigen[63]**.** Presentation of self-peptides by activated DCs may pose a potential threat by inducing auto-immunity, and it is largely unknown how our immune system, despite this potential problem, is capable to avoid activation of self-directed T-cells. The notion that the peptidome presented by activated DCs represents a “snapshot” in time of the environment from which the activation stimulus was acquired partly resolves this question. The mechanisms underlying this snapshot

comprises transient up-regulation of MHC class II synthesis upon activation followed by a shut off of MHC class II synthesis, as well as a change in sorting of MHC class II in endosomes from lysosomal to plasma membrane targeting, resulting in a dramatic and selective increase in half-live of MHC class II molecules that are synthesized directly after DC activation[46,51]. Furthermore, as described above, phagosomes have the capacity to function autonomously, resulting in a preference for presentation of antigens deriving from phagosomes ligands[52,54]. Other evidence supporting this mechanism comes from studies describing that TLR2[64] and TLR4[65,66] can be recruited to and signal from phagosomes. Moreover, selectivity in antigen presentation is increased by the formation of peptide-MHC class II microdomains in the phagosomes, which are presented as such on the plasma membrane of DCs[67]. Finally, peptide-MHC class II from pathogen-containing phagosomes may be transferred in a polarized fashion towards specific plasma membrane domains, such as the immunological synapse between DCs and T-cells[68,69]. In this thesis, we investigated an additional potential regulatory mechanism, involving exosomes. We propose that antigen presenting exosomes are formed in and secreted by phagosomes, resulting in selective presentation by MHC molecules loaded with phagocytosed antigens relative to self-antigens.

2 Extracellular vesicles

The term “extracellular vesicles” (EVs) is a generic term for lipid bilayer enclosed particles that are released by cells. During the last two decades, it became clear that all cell types release EVs, which in many cases were demonstrated to serve intercellular communication[70]. EVs comprise microvesicles (MVs), which bud directly from the plasma membrane, and exosomes, which originate as intraluminal vesicles (ILVs) in multivesicular bodies (MVBs) and are released upon fusion of MVBs with the plasma membrane (**Figure 2**). Apoptotic bodies may be considered as a specific subtype of MVs, but these are released only by cells undergoing programmed cell death (Akers 2013, Yanez-Mo 2015). EVs carry a range of different signaling molecules, e.g. proteins, RNA and lipids[71,72]. As a result, EVs have the capacity to influence the behavior of target cells, including immune cells[73–75]. EVs from diseased cells may play detrimental pathological roles, as has been demonstrated for example for EVs from cancer cells that can tolerize the immune system for their originating cancer cells and facilitate metastasis[76–78].

EVs are thus important means of intercellular communication, both in health and disease. They range in size from 30nm to 100 nm for exosomes and 50 nm to 1000nm for MVs. Depending on their cellular and subcellular origin, EVs are highly heterogeneous with respect to their cargo[79]. Despite this heterogeneity, some proteins, including the

tetraspanins CD9, CD63 and CD81 as well as TSG101, flotillin, and syntenin, appear to be more or less ubiquitously present, and can thus be considered as general EV-markers[80–82]. Proteomic analyses of EV-subsets, derived from cultured cells, that were separated by size, buoyant density, and/or surface markers, confirmed that distinct subsets of EVs with overlapping but distinct protein signatures can be released by one given cell type[82,83]. Moreover, the status of cells also influences the release of EVs and cargo incorporation. For example, the maturation status of DCs dictates the protein composition of the EVs that are released, as determined by proteomics[84], and by the *in vitro* biological effects that these EVs display on T-cells[74,84]. In this thesis, we focus on the putative triggers for the release of antigen presenting EVs by DCs.

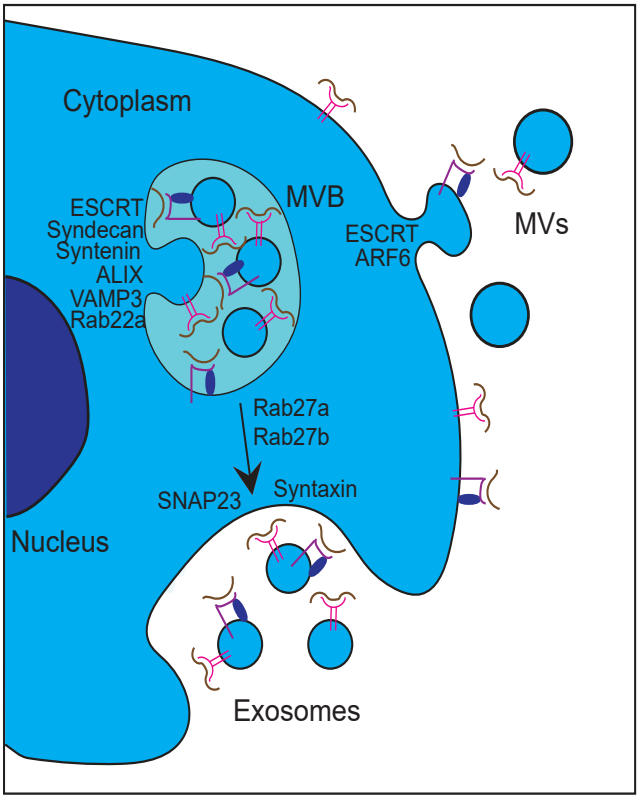

Figure 2: Release of exosomes and microvesicles (MVs. MVs bud directly from the plasma membrane in a process facilitated by ESCRT components. Selection of cargo into MVs involves both ARF6 and tetraspanins. Exosomes are formed as ILVs in MVB. Formation of these ILVs relies on ESCRT components as well as on the syndecan-syntenin-ALIX axis. The latter also drives the selection of cargo, together with tetraspanins. MVB-trafficking towards the plasma membrane is driven by Rab27a, Rab27b, and other Rab family members (not shown). Fusion of MVBs with the plasma membrane, results in the release of ILV as exosomes and is driven by the SNARE molecules syntaxin and SNAP23.

2.1 Biogenesis of exosomes and cargo selection (in DCs)

Dependent on the status of their originating cell, different types of proteins, lipids and RNA species are available for inclusion in exosomes[83,85–88]. The formation of exosomes as intraluminal vesicles (ILVs) at MVB, is dependent on the ESCRT (endosomal sorting complexes required for transport) machinery. ESCRT is composed of four cytosolic protein complexes, designated ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III, which are differentially involved in selecting cargo as well as membrane budding and scission of ILVs away from the cytosol into the MVB[89–91] (**Figure 2**). Membrane proteins that are ubiquitinated at their cytoplasmic domain can be recognized by the ESCRT machinery and targeted for lysosomal degradation via an MVB-pathway[92,93]. For sorting into exosomes, however, ubiquitination of at least some membrane proteins, including MHC class II[94], seems to be dispensable. Although MVB-formation in the absence of ESCRT cannot be excluded[95], efficient formation and release of exosomes does rely on at least some ESCRT proteins, possibly through their generic functions in vesicle budding and specific interactions with accessory proteins that drive protein-sorting[90]. Indeed, efficient secretion of MHC class II-carrying exosomes by DCs relies on the ESCRT-0 proteins Hrs and STAM-1, as well as the ESCRT-I protein TSG101, but not many other ESCRT-components[96,97]. Of note, in B-cells, trafficking of MHC class II at MVBs into exosome precursor ILVs is regulated by incorporation into a web of tetraspanin proteins[98,99]. Tetraspanins are well known to act as chaperoning proteins throughout the endosomal system as well as on the plasma membrane, and are highly enriched in exosomes[100]. At the plasma membrane, tetraspanins form protein domains that are important for cell-adhesion, cell migration, and signaling[81]. Tetraspanins interact with each other as well as with other proteins and lipids. Some family members, including CD63, are thought to be involved in exosome cargo selection[81] (**Figure 2**). Because of their many different functions, tetraspanins may, in addition to cargo selection, also play a role in the functionality of EVs after their release. Other molecular mechanisms associated with EV-cargo selection include the syndecan-syntenin-ALIX protein axis, ARF6, VAMP3 and Rab22a[90,101–104] (**Figure 2**). In conclusion, sorting of cargo in ILVs involves complex mechanisms which are only partly understood. The precise mechanisms for sorting of proteins that are relevant to the immune response, such as MHC complexes, into EVs remain ill defined. Most likely, multiple mechanisms are involved in parallel, which, depending on the cell-type and status, determine the formation of different types of exosomes[90,105].

2.2 Release of exosomes

The majority of MVBs are destined to fuse with lysosomes, ultimately resulting in degradation of the ILV-content by lysosomal hydrolases. Alternatively, ILVs are

released as exosomes into the extracellular milieu when MVBs fuse with the plasma membrane instead. MVBs that are destined to release their content as exosomes may originate by virtue of different sorting machineries as compared to lysosome targeted MVBs. For example, while MHC class II is destined for lysosomal degradation through its ubiquitination and recruitment of this tag by the ESCRT machinery[49], sorting into MVBs carrying exosome-precursor ILVs is independent of ubiquitination[94]. The precise mechanism underlying the latter process remains unclear, although interference with lysosomal acidification was demonstrated to increase exosome release[106–110]. Furthermore, it is hypothesized that MVB that form independently of ESCRT, but instead rely on the syndecan-syntenin-ALIX pathway, escape lysosomal targeting[90].

Five Rab GTPase protein family members were demonstrated to be involved in exosome release[111]. Of these, Rab27a and 27b were most prominently shown to be committed to stimulate MVB-migration towards and docking at the plasma membrane[111] (**Figure 2**). Knockdown of Rab27a interfered with the release of only a specific subset of EVs[76,87], again suggesting the existence of parallel mechanisms for exosome biogenesis and release. Functionally, Rab27a was recently described to be upregulated in a subset of melanomas, inducing the release of EVs by these transformed cells[76]. Also Rab11a, Rab35 as well as Rab7 have been implicated, either directly or indirectly, in the release of exosomes[89,90,112]. The final step in exosome release involves the fusion of the MVB-delimiting membrane with the plasma membrane, which is driven by SNARE proteins, specifically SNAP23 and syntaxin-4[113] (**Figure 2**). Interestingly, expression of SNAP23 is induced by TLR4 signaling, potentially explaining the increase in EV-release in response to DC activation[114]

2.3 Bioactivity of EVs

After their release, EVs can travel large distances in body fluids before being captured by target cells[115], but in many cases bind cells in the vicinity of their release site[112], or even remain bound to the plasma membrane of the parental cell via tetherin[107]. Binding to target cells is determined by the availability of binding factors on the EVs and on the target cells[116].EVs from activated DCs, but not immature DCs, contain the adhesion molecule ICAM-1, which is promoting their binding to T-cells carrying LFA-1 in an active conformation[117,118]. Moreover, cognate interactions between TCRs and cognate peptide-MHC complexes and costimulatory molecules on EVs from DCs may facilitate their recruitment by T-cells[94]. Alternatively, DC-derived EVs can bind other APCs via ICAM-1, lactadherin, tetraspanins and phosphatidylserine that are present on the EV-membrane[119]. Upon binding target cells, EVs may remain attached to the plasma membrane, or they are incorporated in the target cell, either by direct fusion with the

plasma membrane, or via endocytic processes[116,120]. Of note, the status of the acceptor cell can influence the efficiency with which they take up EVs; immature DCs are more efficient in the uptake of EVs compared to mature DCs, on which EVs are retained on the cell surface[121]. After endocytic uptake, EVs may fuse with the endosomal membrane, thus delivering their contents, including RNA, into the cytosol of the target cell[89].

The potency of EVs in antigen presentation was first reported in 1996, when Epstein-Bar virus-transformed B-cells were found to secrete EVs that could present peptide-MHC class II complexes to antigen-specific T-cells[122]. Later, also DCs were described to release antigen-presenting EVs[123]. *In vitro* tumor peptide-pulsed EVs, priorly isolated from murine DCs, could suppress growth of established murine tumors *in vivo* in a CTL-dependent manner[123]. Importantly, only EVs derived from mature DCs could induce cognate T-cell activation[124,125]. Furthermore, while EVs from DCs have the capacity to activate primed T-cells on their own, EV-mediated activated of naive CD4+ T-cells by EVs is greatly facilitated by the concomitant presence of activated bystander APCs[124]. Interestingly, endocytic receptors on DCs can divert endocytosed antigens into highly immunogenic exosomes[126].

Depending on the status of their originating cell, DC-derived EVs can also have tolerogenic effects and prevent the rejection of allografts[73,127,128]. Such observations indicate that DC-derived EVs may provide promising tools in vaccination as well as therapeutics in different clinical settings[129–132]. To this date, multiple clinical trials have been performed to evaluate the clinical potential of DC-derived EVs as immune-based anti-cancer therapy[125,133–135]. Thus far, these clinical trials failed to demonstrate strong anti-tumor immune responses in cancer patients, although administration of tumor-peptide-loaded EVs from *in vitro* expanded DCs was well tolerated[135]. More knowledge is required regarding the mechanisms of formation, release and function of EVs from human DC-to understand their physiological role in antigen presentation and to use them to their full potential as therapeutics.

3. Aim and scope of the thesis

In section 1.3 of this introduction, I posed the main question of this thesis: how is the activation of self-reactive T-cells prevented, while activated DCs present both non-self and self-peptides. In the second section of this introduction, EVs were introduced as players in antigen presentation. To illustrate their role, I explained the mechanisms underlying the release of EVs and introduced their functionality as antigen-presenting entities. The main hypothesis of this thesis (**Figure 3**) is based on this information. We

hypothesize that DCs supplement the peptidome presented on their plasma membrane with exosomes that are selectively enriched for MHC molecules displaying pathogen-derived peptides. In this scenario, DC-derived EVs promote activation of cognate T-cells via clustering of cognate MHC-peptide complexes, thus forming local domains displaying high concentrations of both pathogen-derived peptides as well as costimulatory molecules. DC-derived EVs may either remain attached to the plasma membrane of their originating cell or be released in the extracellular milieu, from where they can be recruited by bystander APCs, endowing these APCs with patches of MHC molecules that are selectively enriched for pathogen-derived peptides, increasing the chance for their recognition by cognate T-cells. We postulate that selective enrichment of MHC molecules loaded with pathogen-derived peptides is established, through preferential generation and release from pathogen-containing phagosomes. DCs contain hundreds of endocytic compartments, that all contain MHC molecules which are in majority loaded with self-peptides and, in case of DC-activation, are subsequently expressed at the plasma membrane. However, only MHC molecules residing at phagosomes can be expected to be loaded with pathogen-derived peptides. Upon formation of ILVs which are destined to be released as exosomes, MHC molecules present on these ILVs can thus be expected to be preferentially loaded with pathogen-derived peptides.

In this thesis we investigated which physiological stimuli could trigger DCs to release EVs capable of inducing adaptive immune responses. In our *in vitro* experiments, we used human peripheral blood monocyte-derived DCs as model cells, because the number of pDCs and cDCs present in peripheral blood did not meet our needs. Moreover, of the three DC-subsets described above, moDCs most aptly resemble inflammatory DCs, as they too derive from monocytes[16,17].

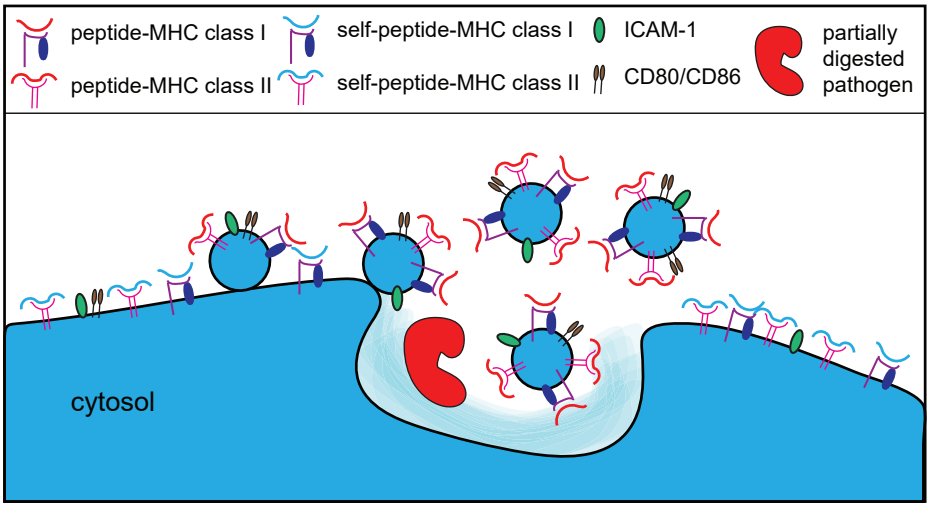

Figure 3: Schematic representation of the main hypothesis. Regurgitation of phagocytosed pathogens from autonomously signaling phagosomes coincides with the release of exosomes that are enriched for MHC complexes carrying pathogen-derived peptides (in red) and also carry costimulatory- as well as adhesion molecules. These exosomes can remain attached to the plasma membrane, thus forming patches with a high concentration of antigenic peptides in combination with costimulatory molecules. Alternatively, exosomes are released in the extracellular milieu, from where they can be recruited by bystander APCs. MHC complexes that are loaded with self-peptides (blue) in other endocytic compartment are preferentially presented at the plasma membrane.

We first set out to review current knowledge on antigen presentation by DC-derived EVs and describe future opportunities and challenges in research on antigen-presenting EVs (**chapter 2)** we summarize the up-to-date knowledge We have previously established that cognate interactions between activated T-cells and DCs stimulate the release of DC-derived antigen-presenting EVs[94]. Based on these observations, we investigated the effect of non-cognate interactions between activated T-cells and DCs on the release of DC-derived EVs. In **chapter 3** we describe the so far unknown effects of activated and non-activated T-cells on the phenotype of pre-activated DCs and the EVs released by these DCs. We found that activated T-cells stimulated pre-activated DCs to develop an even more “mature” phenotype. Moreover, these stimulated DCs released EVs with superior T-cell activating capacities, as determined by *in vitro* activation of cognate T-cells. Based on these findings we propose that, at inflammatory conditions, activated bystander T-cells stimulated further maturation in pre-activated DCs, resulting in the release of EVs, which then efficiently activate third-party cognate T-cells.

Next, we set out to test whether EVs can be released from pathogen-containing phagosomes. This process would involve fusion of phagosomes with the plasma membrane. In **chapter 4,** we are the first to describe that DCs are able to expulse previously phagocytosed bacteria, indicating that fusion of phagosomes with the plasma membrane indeed takes place. Furthermore, we found that during the timeframe of the process of regurgitation, secretion of EVs carrying MHC class I and II was increased. Together, these findings confirm that DCs are capable of releasing phagosomal contents together with EVs, putatively exosomes. Although not yet demonstrated, these observations suggest that exosomes released by phagosomes may be preferentially enriched for MHC molecules carrying pathogen-derived peptides.

In **chapter 5**, we take a side step from DC-derived EVs, and present a mechanism underlying the induction of auto-immunity by self-antigen presentation. Finally, in **chapter 6** I summarize our findings and compare them with published data, culminating in a hypothesis describing how DC-derived EVs can play an essential role in activation of the adaptive immune system while preventing auto-immunity.

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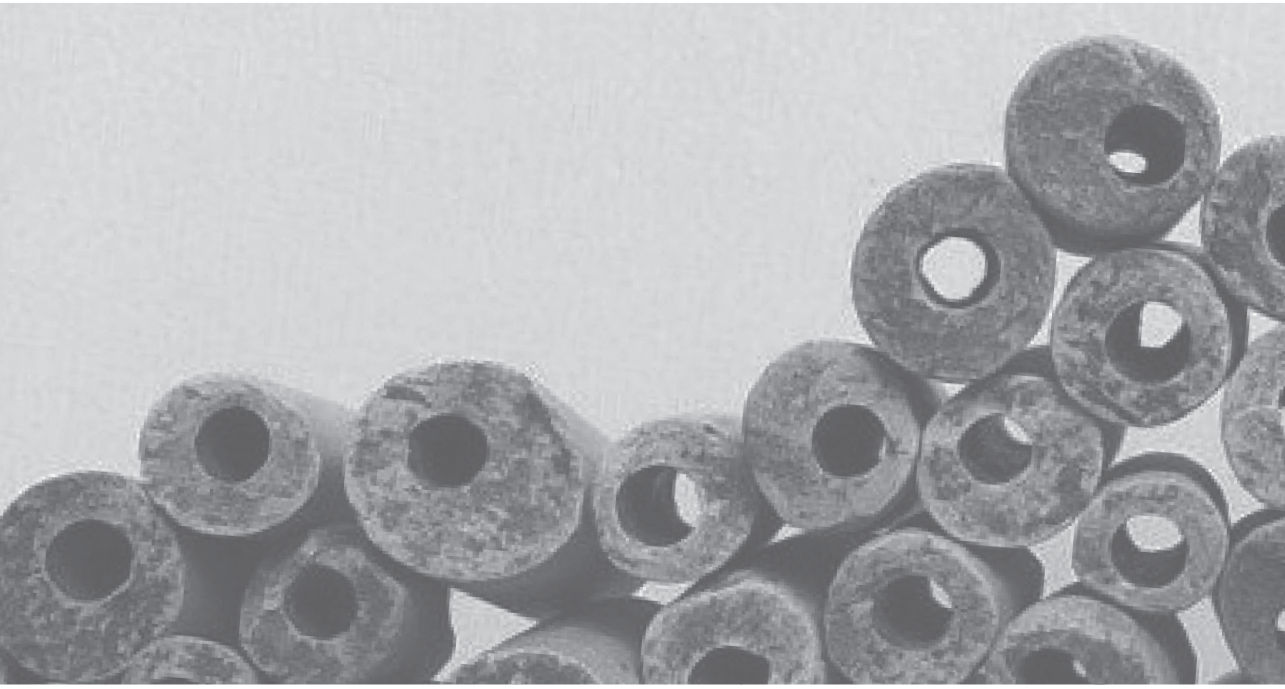

2

Antigen presentation by extracellular vesicles from professional antigen-presenting cells

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Annual Review of Immunology. 2018. 36:435-59.

Abstract

The initiation and maintenance of adaptive immunity require multifaceted modes of communication between different types of immune cells, including direct intercellular contact, secreted soluble signaling molecules, and extracellular vesicles (EVs). EVs can be formed as microvesicles directly pinched off from the plasma membrane or as exosomes secreted by multivesicular endosomes. Membrane receptors guide EVs to specific target cells, allowing directional transfer of specific and complex signaling cues. EVs are released by most, if not all, immune cells. Depending on the type and status of their originating cell, EVs may facilitate the initiation, expansion, maintenance, or silencing of adaptive immune responses. This review focusses on EVs from professional antigen-presenting cells, their demonstrated and speculated roles, and their potential for cancer immunotherapy.

Keywords*: exosomes, microvesicles, MHC molecules, dendritic cells, B cells, T cells*

Introduction

Adaptive immunity is required when innate immunity alone is insufficient to eliminate invading pathogens or to eradicate pathogen-infected or neoplastic cells. At the same time, healthy cells, commensal bacteria, and other nonhazardous environmental compounds should be tolerated. Discrimination between good and bad requires a high level of selectivity in antigen determination, which is dictated by diverse layers of communication between different immune cells. Communication mechanisms include direct cell-to-cell contact, as exemplified by the formation of immune synapses between antigen-presenting cells (APCs) and T cells. Within the immune synapse, major histocompatibility complex (MHC) molecules that are loaded with peptide antigen (pMHC) may bind to cognate antigen-specific T cell receptors (TCRs). Depending on local availability of costimulatory membrane proteins and cytokines, antigen-specific pMHC-TCR interactions may then either stimulate or inhibit T cell proliferation and differentiation. A novel and perhaps even more complex mode of immune regulation is provided for by extracellular vesicles (EVs) that are released by dendritic cells (DCs), B cells, T cells, and other cell types that function within the adaptive immune system. EVs are released by most, if not all, cell types, and depending on their origin they display a plethora of functions (1). First, it was reported in 1996 that human B cell– derived EVs could effectively present MHC class II (MHC-II) peptide complexes (pMHC-II) to CD4 T cells in vitro (2). Later, Théry and coworkers (3) found that injecting mice with pMHC-II-bearing EVs also induced in vivo effects, resulting in the activation of antigen-specific naive CD4 T cells. Zitvogel and coworkers (4) demonstrated that murine bone marrow–derived DCs secreted EVs carrying MHC-I,MHC-II, and T cell costimulatory molecules. When MHC-I molecules on EVs from cultured DCs were loaded with tumor-specific peptides, they could prime tumor-specific cytotoxic T lymphocytes (CTLs) and suppress tumor growth in vivo. Together these three landmark studies have set the stage for many researchers to investigate the role and possible applications of EVs in immunotherapy. Realization that EVs have complex functions in adaptive immunity was further reinforced by observations that selective sets of RNA, including miRNA, are present in EVs from immune cells (5, 6). However, it should be noted that many miRNAs that are found extracellularly are not incorporated in EVs and that probably only a restricted subset of EVs contains biologically significant amounts of miRNA (7). During the last two decades, and after some initial skepticism, it has been increasingly recognized that EVs play a role not only in antigen presentation but also at other levels of communication between immune cells, as illustrated by the >1,000 published research articles on the subject. Although the importance of EVs in antigen presentation is now generally acknowledged and some mechanistic aspects of antigen presentation

by EVs have been disclosed, many regulatory functions of EVs and their discriminatory roles in adaptive immunity remain to be resolved.

EVs comprise at least two classes: microvesicles, which bud directly from the plasma membrane, and exosomes, which are secreted as a consequence of the fusion of multivesicular endosomes (MVEs) with the plasma membrane (**Figure 1**). Exosomes have diameters ranging 30– 150 nm and are on average smaller than microvesicles (100 nm to >1 μm), but their overlapping size distributions and the absence of discriminatory molecular markers often hinder the assignment of isolated EVs to one of these classes in experimental settings (1, 8, 9). Furthermore, the precise molecular machineries that drive EV formation or incorporation of their contents have not been fully resolved (10). As a consequence, studies on the distinctive physiological roles of exosomes and microvesicles are hampered by limited availability of molecular intervention methods. Nevertheless, great progress has been made in recent years. In this review, we use the term EV unless the classification of microvesicle or exosome is evident. We do not aim to extensively evaluate current knowledge on generic mechanisms for molecular sorting into EVs or EV release, as these have been reviewed elsewhere (1, 11). Instead we expand on excellent previous reviews (12, 13), focusing on currently available data on the role of EVs in antigen presentation by professional APCs, and finish by discussing the potential of EVs isolated from DCs in immunotherapy against cancer.

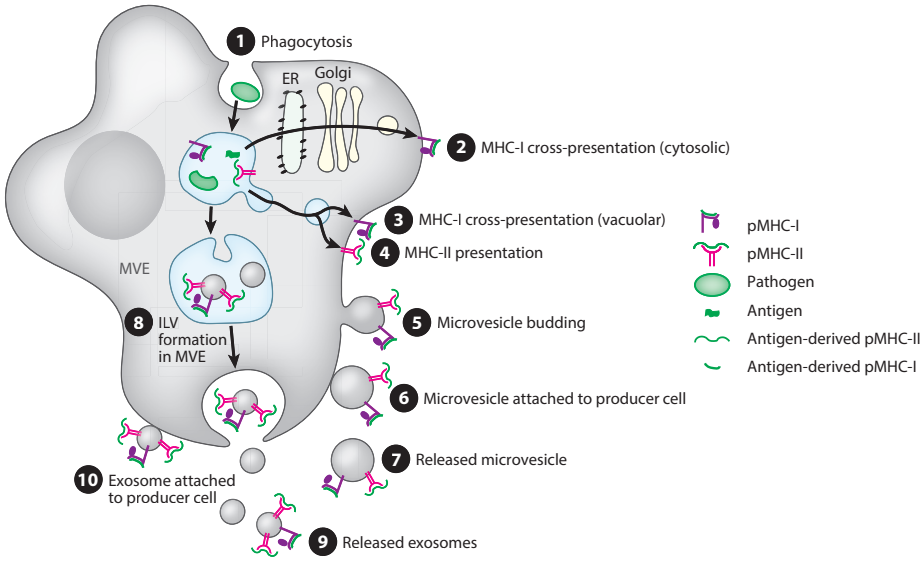

Figure 1: MHC-I and MHC-II pathways in DCs. 1. Phagocytosis and receptor-mediated endocytosis of extracellular material. 2. Cytosolic pathway for pMHC-I cross-presentation at the plasma membrane. 3. Vacuolar pathway for peptide–MHC-I cross-presentation at the plasma membrane. 4. pMHC-II complex formation in endosomes and transfer to the plasma membrane. 5. Microvesicles carrying peptide–MHC-I and peptide–MHC-II complexes budding from the plasma membrane. 6. After budding, microvesicles may remain attached or rebind to the plasma membrane. 7. Alternatively, microvesicles are released in the extracellular milieu. 8. ILVs carrying peptide–MHC-I and peptide–MHC-II complexes are formed within MVEs. 9. Exosomes carrying peptide–MHC-I and peptide–MHC-II complexes are released upon fusion of MVEs with the plasma membrane. 10. Alternatively, exosomes may remain attached or rebind to the plasma membrane after being released by MVEs. Abbreviations: DC, dendritic cell; ER, endoplasmic reticulum; ILV, intraluminal vesicle; MVE, multivesicular endosome.

Intercellular transfer of MHC molecules

The ability of T cells to acquire immune-relevant membrane proteins from APCs, including MHC molecules and costimulatory molecules, both in vitro and in vivo, was reported in the early 1980s (14–16). The transfer of intact pMHC complexes from a donor cell to the cell surface of an acceptor cell is referred to as cross-dressing (17). In contrast to cross-presentation, cross-dressing does not involve loading of acquired antigen onto MHC molecules of the acceptor cell but rather the acquisition of entire pMHC complexes. For example, DCs can acquire pMHC-I complexes from dead or apoptotic tumor cells and present these to naive CD8 T cells, supplementing the cross-presentation pathways (18). Similarly, DCs that were cross-dressed with pMHC-I complexes from virus-infected cells effectively activated previously primed but not naive CD8 T cells, both in vitro and in vivo (19). However, cross-dressing also occurs

between DCs, as clearly demonstrated by in vitro experiments in which mouse DCs acquired substantial amounts of pMHC-I and pMHC-II complexes from cocultured allogeneic DCs (3, 20). Although cross-dressing refers exclusively to the acquisition of pMHC complexes, other membrane-associated molecules can also be transferred in the process (3, 21, 22). One mechanism for transfer has been proposed to involve transient fusions between plasma membrane extrusions of neighboring cells (23, 24). These so-called tunneling nanotubes (TNTs) are continuous plasma membrane bridges, with lengths of up to several cell diameters and thicknesses ranging between 50 nm and 0.5 µm. Depending on the connecting cell types, membrane extensions of opposing cells may either just stick together or be truly fused, connecting the cytosolic compartments of the cells (23, 25). Based on the observation of transfer of fluorescence-tagged MHC-I between HeLa cells, it was proposed that such plasma membrane bridges are important for exchange of MHC-I between cells (26). In a later study, TNTs were shown to develop in a CD40L-dependent manner between human mature DCs, as well as between DCs and CD4 T cells (27). The authors who reported this study proposed a function of TNTs in MHC cross-dressing, which may indeed explain how non migratory lymph node–resident DCs can acquire pMHC molecules from migratory DCs (28, 29). In this scenario, integral membrane proteins such as MHC-I and MHC-II would be transferred by lateral diffusion along a continuous intercellular nanotubular membrane. Alternatively, transfer could occur by means of EVs that after their release remain associated with the plasma membrane of the donor cell and "surf " along the outside of the nanotube to an adjacent acceptor cell. Such a process would be analogous to the transport of membrane viruses such as influenza, which also surf along the exoplasmic side of TNTs for spreading to neighboring cells (30, 31). Indeed, this could explain the punctate rather than diffuse pattern of cross-dressed MHC on acceptor cells, as observed using fluorescence microscopy (26). An alternative mechanism for cross-dressing is trogocytosis, a process in which cells "gnaw" parts of the plasma membrane from adjacent cells (32). For example, trogocytosis was described for basophils acquiring pMHC-II complexes from cocultured DCs, enabling them to stimulate peptide-specific CD4 T cells (33). Separation of these two cell types by porous filters intervened with transfer; hence, it was concluded that direct cell-cell contact was required. The alternative explanation, however, that EVs were released as a consequence of cell-cell contact and subsequently transferred to the acceptor cell, has not been ruled out. Indeed, many cell types release EVs that carry immune regulatory molecules in response to immune synapsis (5, 8, 34–37), and in some occasions EVs have been demonstrated to be captured directly from the immunological synapse (36, 38). EVs that are released into the immune synapse may constitute either plasma membrane–derived microvesicles (37) or exosomes that are delivered by polarized fusion of multivesicular endosomes with the plasma membrane

at the synapse domain (5, 39). This pathway is consistent with, and parallels, targeted secretory delivery of cytokines into the immune synapse (40). Alternatively, EVs may be released outside the immune synapse, as suggested by increased release of EVs into the culture medium of APCs upon stimulation by cognately interacting T cells (34, 41). In conclusion, transcellular acquisition of membrane (proteins) involves distinct mechanisms that may act in a complementary manner.

Extracellular vesicles from dendritic cells

Mouse bone marrow–derived DCs (BMDCs), human primary monocyte-derived DCs (moDCs), and DC cell lines have all been reported to release EVs (3, 4, 34, 42). In a recent study, moDC-derived EVs were described to be very heterogeneous in size, with diameters ranging from 50 nm to > 400 nm, and they could be fractionated in populations with different but overlapping protein compositions (9). MHC molecules and proteins classically described as exosome markers, such as flotillin 1 and 70-kDa heat shock proteins, were found to be equally present in all isolated EV subclasses, while many other proteins were more selectively enriched to particular subclasses of isolated EVs. The authors who published the study identified exosomes by the high enrichment of the endosomal markers CD63, CD81, syntenin 1 and TSG101, and their relative homogeneous, small size of 50–150 nm. Plasma membrane–derived microvesicles, on the other hand, appeared to contain little if any syntenin 1 and TSG101 and to vary in diameter from 50 nm to > 500 nm. Contributions of exosomes are perhaps most convincingly demonstrated by static immuno–electron microscopy images of human and murine DCs, showing MVE–plasma membrane fusion profiles releasing 60 to 100 nm exosomes carrying MHC molecules and exosome-characteristic tetraspanin molecules such as CD63, CD81, and CD9 (4, 34). Both immature and activated DCs release EVs, but their number and cargo composition are dictated by the status of the DC and interactions with T cells. For example, EVs from mature DCs are relatively enriched for CD86 and intercellular adhesion molecule 1 (ICAM-1), whereas EVs from immature DCs carry more milk fat globule–epidermal growth factor–factor VIII (MFG-E8) (43). These differences are partly due to changing protein expression profiles, but also to changing subcellular protein distributions during DC maturation. For instance, MHC-II is redistributed from endosomes to the plasma membrane during DC maturation (44), and its relative abundance in exosomes versus microvesicles can thus be expected to shift accordingly. Moreover, the relative contributions of exosomes and microvesicles to the total EV population are likely to change during DC maturation (9).

In response to cognate interactions with CD4 T cells, activated DCs develop a mechanism by which MHC-II is sorted independently of β-chain ubiquitination to the intraluminal

vesicles of a distinct subclass of MVEs, to be secreted in association with CD9-carrying exosomes (34). Consistent with this observation, sorting of MHC-II in MVEs to exosomes appears to rely on only a few components of the endosomal sorting complexes required for transport (ESCRT) machinery, including TSG101 (45, 46). The ESCRT mechanism is also required for formation and pinching off of microvesicles at the plasma membrane, however, and intervention with this complex can therefore not be used to discriminate exosomes from microvesicles (13, 39, 46). Exosome secretion was more specifically inhibited by knocking down the GTPases Rab27a and Rab27b (47). Interestingly, the release of EV-associated MHC-II by immature DCs from Rab27a/Rab27b double knockout mice was hardly affected (47), and this may indicate that the majority of EV-associated MHC-II from immature DCs is associated with plasma membrane–derived microvesicles rather than with exosomes. In contrast to immature DCs, DCs that were activated by lipopolysaccharide (48–50) or through cognate interactions with CD4 T cells (34) released significantly more EVs compared to immature DCs, and these EVs have the characteristics of exosomes (34). Together, these types of experiments are indicative of two distinct MVE pathways, one for lysosomal targeting and the other for exosome secretion, with the first being particularly dominant in immature DCs and the second in activated DCs. Activated DCs, however, also release microvesicles, as exemplified by the release of IL1-β-containing microvesicles by lipopolysaccharide-activated DCs, in response to stimulation of the P2X7 receptor by extracellular ATP (51). DC-derived EVs are reported to have either immune-inhibitory or -stimulatory functions, depending on the status of the donor DC (discussed in detail below). A complication of the molecular and functional analyses of DC-derived EVs is that it is extremely difficult, if not impossible, to obtain EVs from populations exclusively comprising only immature DCs or only activated DCs. Cultures of non stimulated DCs always contain some activated DCs, as determined by the expression of activation markers. Conversely, DC stimulation with Toll-like receptor (TLR) ligands usually results in asynchronous and incomplete activation of cells within the population. It is thus very likely that EVs isolated from either immature DCs or activated DC cultures are always contaminated to some extent by EVs from DCs with the opposite status. Therefore, it should be kept in mind that such contaminating EV types, even when present in relatively low quantities, may significantly influence functionality tests.

Human blood has been estimated to contain ~10^5 EVs per microliter, and most of these derive from platelets or erythrocytes (52). Multiple EV markers can be detected in EVs isolated from blood, but the concentration of MHC molecules is very low (53) (X. Zhang & W. Stoorvogel, unpublished data), suggesting either that APCs release relatively low amounts of EVs into circulation or that EVs are rapidly removed by other cells after

release. When EVs isolated from DCs or B cells were injected into mice, they were able to interact with cells far away from the injection site (54, 55). Nevertheless, it is likely that most EVs secreted in situ by APCs interact with nearby cells, immediately after their release. This idea is supported by the demonstration that migratory DCs home to lymphoid organs, where locally released EVs can be efficiently recruited by interacting or nearby lymph node–resident DCs (28, 56). In contrast, EVs that were isolated from cultured DCs and introduced into the circulation were inefficient in stimulating T cell responses in vivo (56), supporting the notion that efficient immune responses to EVs from migratory DCs require local secretion in lymphoid tissues.

Antigen presentation by MHC-I and the role of dendritic cell-derived extracellular vesicles

MHC-I molecules function on all nucleated cells to bind and display protein-derived peptides to CD8 T cells. When cells are infected or transformed, a fraction of their MHC-I molecules willdisplay foreign peptides, and recognition of such complexes by CD8 CTLs via peptide-specific cognate TCRs results in selective killing of these infected or transformed cells. The majority of peptides that bind to MHC-I are generated from proteins that are endogenously expressed in the cytosol. Cytosolic proteins can be degraded by the proteasome and the resulting peptides trimmed by aminopeptidases. After being translocated from the cytosol into the endoplasmic reticulum (ER) by a transporter associated with antigen processing (TAP), peptides can be loaded onto newly synthesized MHC-I molecules. The resulting pMHC-I complexes are then transported along the secretory pathway toward the plasma membrane for display to CD8 T cells. When cells are infected with viruses or bacteria, or transformed by mutation, their MHC-I molecules will present, in addition to endogenous peptides, peptides from pathogen-encoded or tumor cell–specific proteins, thus enabling recognition and eradication by antigen-specific CTLs. To stimulate the generation of CTLs with specificities toward relevant antigens, cognate naive CD8 T cells need to be activated by DCs. DCs have the unique ability to present peptides derived from proteins of exogenous origin that are acquired by endocytic processes. This process of cross-presentation of exogenously acquired antigen involves both vacuolar and cytosolic pathways (steps 2 and 3 in **Figure 1**), and their relative contributions may differ between DC lineages (17, 57). In the cytosolic pathway, endocytosed proteins are translocated across the endosomal membrane into the cytosol for degradation by proteasomes. For loading onto MHC- I molecules the resulting peptides are transported by TAP, either into the lumen of the ER or, uniquely for DCs, into the lumen of endosomes or phagosomes. In the alternative vacuolar pathway, endocytosed proteins are directly degraded by lysosomal proteases to generate peptides that are loaded onto MHC-I in the endosomal

or phagosomal lumen (17, 57–59). When resting DCs present antigens in the absence of costimulatory molecules, corresponding cognate CD8 T cells may become anergic (60).

The contribution of DCs to the generation of self-tolerance has been termed cross-tolerance (61). In the presence of danger signals, however, cross-presentation by activated DCs is considered to represent a major pathway for the activation of naive CD8 T cells (57). Thus, DCs are essential to initiate and control proper cellular adaptive immunity through selective activation of pathogen- or tumor-specific naive CD8 T cells.

In their early study, Zitvogel and colleagues observed that EVs isolated from cultured immature mouse DCs could prime CD8 T cell responses, both in vitro and in vivo (4). EVs isolated from activated human moDCs could stimulate peripheral CD8 T cells in vitro, independently of target DCs, demonstrating an intrinsic stimulatory capacity of EVs (62). However, it should be noted that in the absence of acceptor DCs, EVs may activate only primed T cells and not naive T cells. In vitro activation of mouse CD8 T cells by EVs carrying cognate pMHC-I complexes was greatly supported by bystander DCs. For example, the immunological efficacy of cross-dressing by EVs was shown in experiments where pMHC-I complexes transferred by EVs isolated from cultured human DCs could provide naive DCs with the capacity to efficiently prime melanoma-specific CD8 T cells in HLA-A2 transgenic mice (63). Cross-dressing of MHC-I by means of intercellular transfer of EVs can be expected to occur most effectively at sites with a high density of active immune cells, such as in the spleen and lymph nodes. Indeed, primary MHC-I-restricted CD8 T cell responses were most efficient when DC-derived EVs were transferred in vivo onto mature DCs in lymphoid tissues, in combination with CpG adjuvants (64). But the status of the EV-producing DC is also important, as EVs from mature DCs could more strongly stimulate CD8 T cell proliferation in vitro and in vivo than could EVs from immature DCs, and more efficiently induce antigen-specific CTL responses, CD8 T cell memory, and antitumor immunity (65, 66). In vivo mouse experiments demonstrated that transfer of pMHC-I complexes predominantly occurs in a unidirectional manner from migratory DCs toward lymph node– or spleen-resident DCs (28). Cross-dressed resident DCs were in fact more efficient in activating CD8 T cells than the pMHC-transferring migratory DCs (29), indicating a prominent role for cross-dressing. Together, these findings are consistent with the idea that activated DCs endocytose antigen in peripheral tissues,cross-present antigens onto MHC- I molecules, and migrate to lymphoid tissues, where they release EVs carrying pMHC complexes and costimulatory molecules for cross-dressing of interacting or nearby lymphoid tissue–resident DCs (**Figure 2*a*–*c***).

Antigen presentation by MHC-II and the role of dendritic cell-derived extracellular vesicles

In contrast to MHC-I, MHC-II is constitutively expressed only by professional APCs, including DCs, B cells and macrophages, and some epithelial cells. Newly synthesized MHC-II molecules are transported from the ER to endosomes with their peptide-binding groove occupied by the molecular chaperone invariant chain (CD74).Within endosomes, the invariant chain is degraded and the resulting class II–associated invariant chain peptide (CLIP) is subsequently displaced, allowing other peptides to bind to MHC-II. MHC-II-bound peptides mostly derive from proteins that are degraded by lysosomal proteases within the endocytic pathway. The originating proteinsare either expressed by the APC itself and transported to endosomes and lysosomes via the vacuolar pathway or derived from endocytosed exogenous molecular complexes or apoptotic cells (fragments) (**Figure 1**). MHC-II-bound peptides can, however, also derive from proteins that are originally expressed in the cytosol, encoded by the APC itself or by an infectious pathogen, and have entered the lysosomal pathway through (micro) autophagy (67). In the absence of danger signals, pMHC-II complexes are ubiquitinated and as a consequence sorted by the ESCRT machinery to intraluminal vesicles of MVEs. When such MVEs fuse with lysosomes, their contents, including the pMHC-II-containing intraluminal vesicles, will be degraded (68). In activated DCs, however, ubiquitination of MHC-II is abrogated, thus allowing efficient redistribution of newly formed pMHC-II complexes from endosomes to the plasma membrane (69). Surface-expressed pMHC-II can then be recognized by naive CD4 T cells that express antigen-specific TCRs (**Figure 2*a***).

Similar to CD8 T cells, peptide-specific CD4 T cells will be activated only when proper co-stimulation is provided by the APC. Naïve T cells that are triggered by cognate pMHC-II complexes on non activated DCs in the absence of costimulatory signals may enter a state of unresponsiveness, or anergy (70). Alternatively, antigen presentation by immature (or resting) DCs may also induce the expansion and differentiation of CD4 T cells toward an immunosuppressive regulatory phenotype (Treg), to control peripheral T cell tolerance and autoimmunity (71). In this case, recognition and trans-endocytosis of DC-encoded costimulatory molecules CD80 and CD86 by CD4 T cell–encoded CTLA-4 plays a major role (21), a process that may involve intercellular transfer of these membrane molecules via EVs.

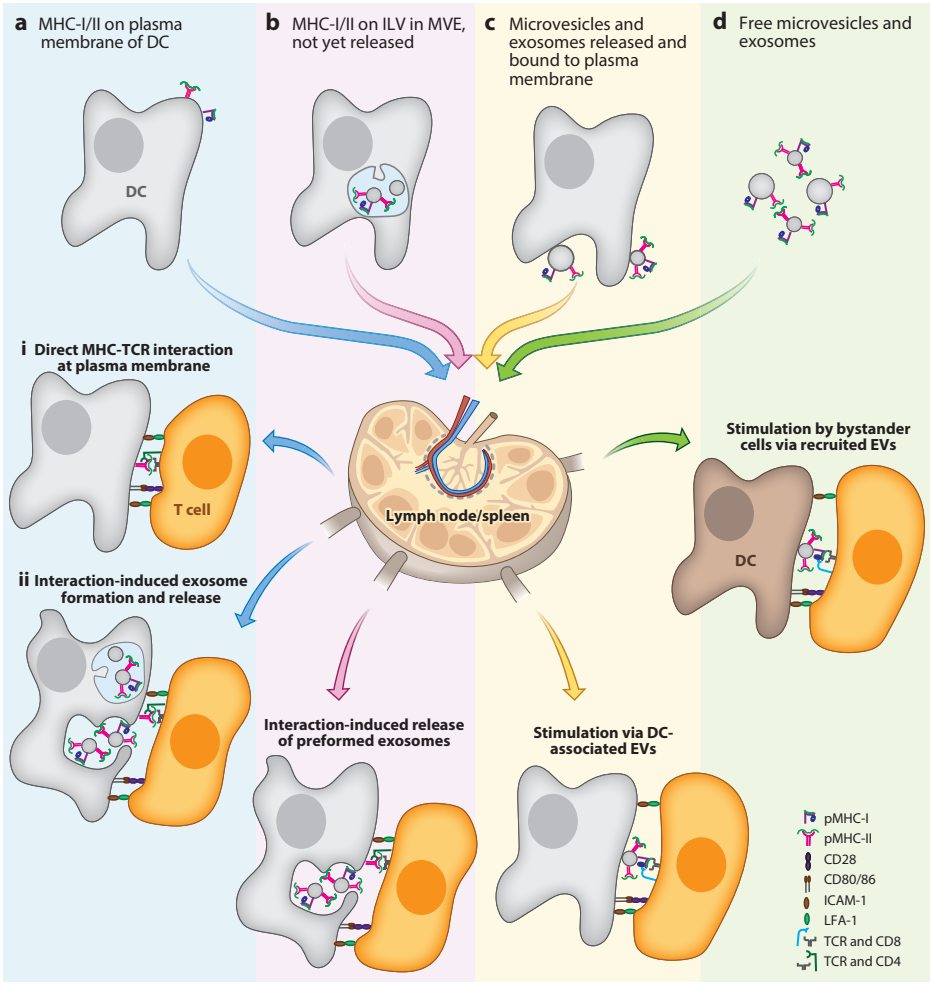

Figure 2: Transfer and interaction of DC-derived EVs with T cells. (*a*) Peripheral DC presenting pMHC complexes at its plasma membrane. (*a*, *i*) After traveling to a lymph node or the spleen, plasma membrane peptide-MHC is presented directly to T cells. (*a*, *ii*) DC–T cell interactions in lymphoid organs may induce the formation and release of EVs (only exosomes and not microvesicles are shown), which may then interact with the T cell or bystander cells. (*b*) Peripheral DC packing peptide-MHC complexes into multivesicular endosomes (MVEs), which release their preformed intraluminal vesicles (ILVs) as exosomes only after the DC reaches a lymph node or the spleen and interacts with a target cell. (*c*) A peripheral DC that has released exosomes and/or microvesicles that remained associated with the plasma membrane of the producing DC while traveling to a lymph node or the spleen. Here the membrane-associated EVs can either present their peptide-MHC complexes directly to T cells or be released and transferred to bystander DCs. (*d*) Free microvesicles that are released by peripheral DCs travel toward a lymph node or the spleen. Here they can activate T cells, either directly or after being recruited by bystander APCs.

When stimulated by cognate pMHC-II in the presence of costimulatory signals, naive CD4 T cells will differentiate into CD4 T helper (Th) cells, which can provide assistance either in the cellular immune response (Th1) by stimulating the activation of macrophages or cytotoxic CD8 T cells, or in the humoral immune response (Th2) by activating B cell class switching. As described above, the outcome of an interaction between DCs and T cells carrying a cognate TCR depends on the activation status of the DC. DC recognition of conserved pathogen-associated molecular patterns or damage-associated molecular patterns induces the expression of costimulatory plasma membrane proteins such as CD80 and CD86 (the ligands of the T cell costimulatory receptor CD28) and CD70 (the ligand for the T cell costimulatory receptor CD27) as well as secretion of proinflammatory cytokines, but also (as discussed below) the release of immune regulatory EVs. Indeed, CD27 co-stimulation of CD8 T cells appears to be a key pathway of CD4 T cell help in CTL responses (72). Alternatively, pMHC-II- and pMHC-I-presenting DCs can be stimulated by cognately interacting, activated CD4 T cells to upregulate CD40 ligand, licensing the DCs to activate CD8 T cells carrying both CD40 and representative TCRs (73). While MHC-I and MHC-II present different sets of peptide antigens to activate CD8 and CD4 T cells, respectively, a shared source of peptides could thus ensure presentation by the same DC, helping the differentiation of effective CD8 T cell responses (74).

Transfer of MHC-II molecules between allogeneic DCs plays a major role in primary mixed leukocyte reactions (75), demonstrating the potency of MHC-II cross-dressing in antigen presentation. After allogeneic DCs were injected into acceptor mice, the majority of draining lymphnode–resident DCs expressed donor MHC-II molecules, and vice versa, demonstrating bidirectional transfer of MHC-II molecules between migratory and resident DCs (76). In vitro and in vivo experiments demonstrated that EVs isolated from antigen-loaded DCs could stimulate both primed CD4 T cells and CD4 T cell clones (3, 42) but were less effective in stimulating naive T cells (3, 77, 78). Activation of naive CD4 T cells was dependent on the additional presence of mature bystander DCs, and co-stimulation by CD80 and CD86 on these DCs (3, 19, 43, 56, 63, 79), indicating that DC-derived EVs can amplify CD4 T cell responses through crossdressing of DCs that have not encountered the antigen directly. In vivo studies confirmed that EVs from antigen-pulsed mature DCs were more effective in providing protection against infections than EVs from antigen-pulsed immature DCs (80, 81). Activated, antigen-loaded, migrating DCs were shown to transfer EVs to spleen-resident DCs, which could then activate CD4 T cells (56) (**Figure 2*d***). EVs from activated DCs are highly enriched in ICAM-1, a ligand for the integrin LFA-1 on DCs and T cells, explaining why they are much more effective in priming T cell responses than EVs from immature DCs (42, 56, 77, 78). CD8 DCs in lymphoid tissue express higher levels of LFA-1 than peripheral CD8− DCs, facilitating

their role as EV recipient in vitro and in vivo (78). DC-derived EVs carry complement (82) and therefore could potentially also be recruited by the complement receptor CD21 on follicular DCs (FDCs). This idea is supported by the observation, reported by the Gabrielsson laboratory, that CD8 T cell activation in response to immunization with EVs from whole protein antigen–loaded DCs, but not from antigen peptide–loaded DCs, was entirely dependent on B cells and complement activation (83). These authors proposed that B cells may recruit EVs loaded with MHC-I- and MHC-II-antigen complexes from DCs to help their transfer to FDCs, facilitating both CD4 and CD8 T cell responses. Later, however, the same laboratory reported that similar immune responses could be obtained with EVs isolated from protein antigen loaded MHC-I knockout DCs (84), indicating independence of the presence of MHC-I on EVs in this setting, and that isolated EVs may also serve as adjuvants. Either way, DCs that were pulsed with intact protein antigen could also stimulate in vivo antigen-specific immunoglobulin responses, in both naive and primed recipients, demonstrating their capacity to induce humoral immunity (48).

In vivo studies have demonstrated that DC-derived EVs can induce humoral immune responses (81, 85). Others reported that injected DC-derived EVs were as effective as their originating antigen-pulsed DCs in inducing both protective humoral and cellular immune responses against the parasite *Toxoplasma gondii* (86, 87). That exosomes secreted in vivo also play a role is supported by a recent study in which *Mycobacterium tuberculosis*–infected DCs from Rab27a-deficient mice were found to be limited in their ability to present antigen to CD4 T cells, suggesting that Rab27a dependent secretion of exosomes by these DCs is important to acquire adaptive immunity against tuberculosis (88).

Upon transfer to acceptor DCs, EVs may either remain associated with the plasma membrane or fuse with the acceptor cells, integrating the membrane proteins and cytosolic constituents of the EVs into the respective compartments of the acceptor cells. The latter was perhaps most convincingly demonstrated by the transfer of EV-contained miRNAs that could repress target mRNAs in acceptor DCs (89). Indeed, distinct sets of miRNAs are incorporated into EVs from immature versus mature DCs and transferred to recipient DCs to modulate immune responses (89, 90). After endocytic uptake of recruited EVs, peptides may be eluted from associated MHC-II and transferred to endogenous MHC molecules (cross-presentation). This process was found to enable acceptor DCs to activate CD4 T cells after the acquisition of EVs from alloreactive DCs (54, 56).

It is also possible that a significant proportion of released EVs remain associated with the plasma membrane of the producing cell itself, by either retention or recapture [**Figures 1** (steps 6–9), **2*c***], although this has not been investigated for DCs. Recapture is likely to occur, as this would be equivalent to the demonstrated recapture by bystander DCs involving ICAM-I binding to LFA-1 (3, 77). Retention is also possible via other mechanisms. For example, exosomes secreted by HeLa cells remained largely bound to the cell surface with the aid of tetherin (BST2) (91). Analogously, the spread of certain enveloped viruses, including HIV, is limited by tetherin-mediated restriction of the virions at the plasma membrane of the virus-producing cell (92). Interestingly, tetherin knockout DCs were compromised ex vivo in their capacity to stimulate antigen-specific CD4 T cells (93) and in vivo to induce an adaptive cell-mediated immune response against retrovirus infection (94). Depending on the stimulus, the expression of tetherin is increased in activated DCs (95). This implies, although direct evidence is lacking, that EVs generated by activated DCs, but less so by immature DCs, may be retained at the plasma membrane of the producing DC by tetherin. Hence it is possible, although entirely speculative at this time, that those EVs that remain associated with the producing DC serve as signaling platforms to DC-interacting cells, for example, through physical clustering of MHC and/or costimulatory molecules. It is also possible that such surface-associated EVs dissociate only in response to DC-interacting cells, ensuring targeted delivery.

Transfer of extracellular vesicles from dendritic cells to other cell types and immune tolerance

EVs derived from DCs with an immunosuppressive phenotype have been shown to decrease inflammation systemically (96–99). IL-10-treated tolerogenic DCs, and their EVs, have a low expression of costimulatory and coregulatory molecules compared to mature DCs and their EVs (97–99). Importantly, EVs from IL-10-treated DCs were shown to delay onset of arthritis and reduce the severity of the disease systemically via an MHC-II-dependent mechanism (99). In transplantation studies, infusion of donor-derived EVs resulted in the induction of regulatory B and T cells, increasing survival of the allograft (100, 101). Also the course of autoimmune disease can be influenced by EVs, for instance in systemic lupus erythematosus, where autoantigen-presenting EVs have been shown to play a role (102, 103). DCs can cross-dress CD4 T cells with pMHC complexes and costimulatory molecules during antigen-specific immune synapse formation (34, 36, 38, 42). In vitro, the release of MHC-II carrying EVs from antigen-loaded DCs was stimulated by cognately interacting CD4 T cells (34). A significant proportion is recruited to the DC-interacting CD4 T cells (33, 34, 41, 42). Like for DCs, transfer to CD4 T cells depended on integrin binding, with LFA-1 on the acceptor cells and ICAM-1 on the EVs

(42, 49, 77, 78, 104). The functions of such antigen-presenting T cells have not been completely clarified. One proposed role is that the transferred pMHC and costimulatory molecules help to sustain the activated status of the T cells, thereby releasing the requirement forfurther activation by DCs (105). Alternatively, they can signal to other cells. For example, CD4 Th1 cells that were cross-dressed with both MHC-I- and MHC-II-antigen complexes were shown to prime effector antigen-specific CD8 T cells in a CD80 dependent manner (38). Furthermore, T cells that were cross-dressed with DC-derived pMHC, costimulatory molecules, and CD40 have been demonstrated to establish CD4 and central memory CD8 T cell responses (106–108). Alternatively, cross-dressed CD4 T cells have been proposed to function in a negative-feedback loop that inhibits further expansion of antigen-experienced CD4 T cells, thereby limiting clonal expansion and allowing recruitment of other naive T cells (109). Also, presentation by CD4 T cells of cross-dressed pMHC-II complexes in the absence of costimulatory molecules may induce apoptosis and hyporesponsiveness of antigen-specific T cells (110). Activated human, but not murine, CD4 T cells have the ability to endogenously express MHC-II, and the peptide repertoire associated with MHC-II on human CD4 T cells overlaps with that of professional APCs (111). MHC-II/antigen presentation by T:T cell interactions induces anergy, and this pathway has been proposed to function in limiting the expansion of self-reactive CD4 T cells (112). Consistent with the recruitment of MHC-II-carrying EVs from cultured DCs to cognately interacting CD4 T cells (34, 42), it is possible that contributions of cross-dressed pMHC-II, in addition to endogenously expressed pMHC-II complexes, also contribute in tolerance induction by antigen-presenting CD4 T cells. Cross-dressing of T cells may also suppress immune reactions by enabling T cells with APC capacities to kill both antigen-presenting DCs and other antigen-presenting T cells (106). Th1 APCs that acquired pMHC-II could induce apoptosis in cognate memory CD4 T cells while leaving the naive CD4 T cells unaffected, thus establishing a more varied TCR repertoire (109). In an experimental autoimmune encephalomyelitis rat model, transfer of EV-associated pMHC-II complexes from APCs to antigen-specific T cells was associated with the induction of peripheral self-tolerance (113).

Self-reactive T cells that escape thymic negative selection can also become anergic or be deleted by peripheral tolerance mechanisms that involve lymph node stromal cells. Lymph node stromal cells include lymphatic endothelial cells, blood endothelial cells, and fibroblastic reticular cells, and all of these are thought to ectopically express peripheral tissue antigens as well as MHC-I and MHC-II (114). The outcome of MHC-antigen presentation is determined by the presence or absence of costimulatory molecules, including CD80, CD86, and programmed death-1 (PD-1). Owing to the absence of costimulatory molecules on lymph node stromal cells, antigen presentation

by endogenously expressed MHC can induce deletion of both self-reactive CD4 T cells and self-reactive CD8 T cells. Cross-dressing of lymph node stromal cells with pMHC-II carrying EVs from DCs also promotes cognate CD4 T cell unresponsiveness and deletion (115). The relative contributions of endogenously expressed MHC-II and cross-dressed MHC-II to lymph node stromal cell–driven CD4 T cell deletion are unclear (reviewed in 116).

Role of extracellular vesicles from B cells in MHC-II antigen presentation

EVs isolated from both human and murine B cell lines carried pMHC-II complexes, as well as costimulatory and adhesion molecules, and could induce pMHC-II-restricted T cell responses in vitro, thus demonstrating for the first time the antigen-presenting capacities of EVs (2). Electron microscopy analysis of Epstein-Barr virus (EBV)-transformed B cells revealed MVEs fusing with the plasma membrane, thus classifying the released EVs as exosomes. pMHC-II in B cell exosomes is incorporated into detergent-resistant protein complexes that also contain CD63 and other members of the tetraspanin family (2, 117–119). It has been proposed that proteins incorporated into a CD63-containing tetraspanin network can be recruited at MVEs by the ESCRT machinery via the cytosolic adaptors syntenin and ALIX for incorporation into exosomes (10, 120–122). To generate antibody-secreting plasma cells, resting immature B cells in germinal centers of secondary lymphoid organs need to be activated consecutively by antigen-carrying FDCs and CD4 T cells to undergo the processes of isotype switching and affinity maturation, proliferation, and differentiation (123) (steps 1 and 3 in **Figure 3**). Antigens that form complexes with antibodies and/or complement are retained by FDCs by their FcRIIb or complement receptor CD21/35 (124). B cells can engage FDC-displayed antigens via their plasma membrane–bound immunoglobulin, the B cell receptor (BCR). Subsequently, this association is enforced by integrin-receptor interactions, establishing an immune synapse between the B cell and the FDC. Antigens can then be released directly from the FDC membrane by the action of lysosomal proteases that are locally secreted into the synapse by the B cell, and be endocytosed by the BCR and transported to endosomes and lysosomes for further degradation (125) (step 1 in **Figure 3**). Resulting peptides are then loaded within the endocytic compartments onto MHC-II, after which pMHC-II complexes can be displayed at the plasma membrane. Such primed B cells are subsequently activated when their surface-displayed pMHC-II complexes are recognized by cognate TCRs on antigen-specific CD4 T cells (126) (step 3 in **Figure 3**). Full B cell activation, however, also requires binding of B cell CD40 to CD40 ligand (CD40L), which is only displayed by CD4 T cells when they have previously been activated by DCs (step 2 in **Figure 3**). In this way, licensing of CD4 T cells by DCs ensures selective activation of antigen-specific B cells to proliferate and differentiate

into high-affinity antibody-producing plasma cells. The release of EVs by primary B cells is induced by pMHC-II ligation with cognate TCR on CD4 T cells (41, 127–129) (step 3 in **Figure 3**) and can amount to as much as 12% of all cellular MHC-II per day (41). T cell–induced release of B cell–derived EVs also requires CD40 and IL4 signaling, and relies on NF-κB (127, 128), sharing the classical signaling pathways required for T cell–induced B cell proliferation. IgG-mediated BCR cross-linking in an immortalized B cell line also stimulated EV release but only to a minor extent (130), suggesting that the TCR–pMHC-II interaction is the major determinant for EV release by B cells. Exosome release is constitutive by human B cells that are infected by EBV (128), explaining high exosome secretion by EBV-transformed cell lines, with an equivalent of up to ~10% of all cellular MHC-II being released in 24 h (2). The EBV oncogene-encoded latent membrane protein 1 (LMP1) mimics downstream signaling via CD40 by constitutively activating NF-κB, explaining how EBV transformation induces exosome secretion by B cells (131). A pathological role for LMP1-carrying exosomes from Burkitt lymphoma B cells is indicated by the observation that they supported in vitro B cell proliferation, differentiation, and immunoglobulin class switching (132). Exosomes from EBV-infected B cells were also found to contain EBV-encoded miRNAs that could be functionally transferred to non-infected DCs, possibly interfering with the development of adaptive immunity (133). One of the potential targets of B cell–derived EVs is FDCs in lymphoid follicles. FDCs do not synthesize MHC-II themselves nor do they have MHC-II incorporated into their plasma membrane, but they are abundantly decorated at their plasma membrane with MHC-II-carrying EVs (134, 135). The notion that these EVs may be largely acquired from B cells was supported by the observation that isolated B cell exosomes were efficiently and preferentially recruited in vitro by FDCs from tonsil lymphoid tissue, as compared to other cell types within the same preparation (135). FDCs are non-phagocytic cells; hence, the residency time of recruited EVs on the plasma membrane may be quite long. How B cell–derived EVs are recruited by FDCs is unknown. However, B cell EVs carry complement component 3 (C3) fragments (136) as well as BCR-antigen complexes (128, 130), and C3 fragments on these EVs were shown to stimulate T cell response in the presence of suboptimal antigen concentrations (136). Therefore, it is likely that FDCs recruit B cell exosomes via their FcR and/or complement receptors. Such binding might be enforced, for example, by integrin α4β1, which is also highly enriched and functional on B cell exosomes (118, 137) and known to play an important role in B cell–FDC interactions in germinal center responses (138). Exosomes from B cells, but also from DCs, are protected from complement-mediated lysis by incorporated glycosylphosphatidylinositol-anchored CD55 and CD59 (139), potentially contributing to their long residency time on FDCs. As explained above, antigen-specific CD4 T cells are essential drivers of B cell affinity maturation and development of memory B cells (140),

and maintenance of DC-primed antigen-specific T cells requires constant stimulation, a task that could be performed by FDC-associated B cell exosomes (step 4 in **Figure 3**). This scenario is supported by the observation that isolated, antigen-loaded B cell exosomes can directly stimulate primed, but not naive, CD4 T cells (41, 141). Exosomes that were isolated from cultured primary B cells and injected intravenously or subcutaneously into mice were captured in the subcapsular sinus of lymph nodes by macrophages, involving binding of exosome-linked α2,3- linked sialic acids to CD169 in the marginal zone of the spleen and lymph nodes (55). InCD169−/− mice, such exosomes were not efficiently retained in the subcapsular sinus and penetrated deeper into the paracortex, increasing the response to antigen-pulsed exosomes (55). These data suggest that functional B cell exosomes are normally released locally within follicles in response to B cell–T cell interactions, and that B cell exosomes released outside the lymphoid tissue are eliminated by macrophages to control immune responses. Another level of control is dictated by upregulation of FasL on exosomes from activated B cells and EBV-transformed B cells (142). The presence of FasL induced apoptosis of antigen-specific CD4 T cells (143), suggesting a negative-feedback-loop to restrain T cell–mediated responses.

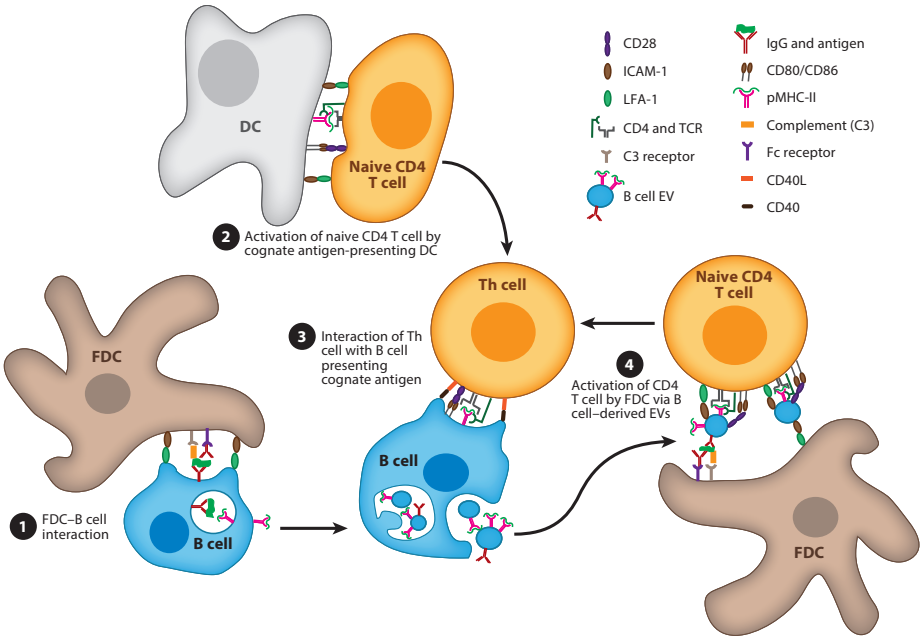

Figure 3 Hypothetical model for the role of B cell exosomes. 1. A B cell interacts with a follicular DC (FDC) from which it obtains opsonized antigens via BCR-mediated endocytosis. Endocytosed antigen is processed into peptides that are presented by MHC-II on the B cell plasma membrane. 2. A naive CD4 T cell is activated by a DC presenting cognate peptide–MHC-II complexes, resulting in T cell proliferation and differentiation into Th cells. 3. A Th cell encounters a B cell presenting cognate peptide–MHC-II complexes, resulting in the B cell releasing exosomes carrying peptide–MHC-II. 4. These exosomes are recruited by FDCs, and in association with these cells they present peptide–MHC-II to CD4 T cells, for further CD4 T cell expansion, differentiation, or silencing.

Antigen presentation by macrophage-derived extracellular vesicles

Macrophages clear their environment from pathogens through phagocytosis and play essentialregulatory roles in innate and adaptive immune responses. Macrophages also function in antitumorimmunity by cross-presenting dead cell–associated antigens to initiate CD8 T cell responses (144). CD8 T cells were much better activated when cocultured together with both splenic macrophages and CD8 DCs, as compared to macrophages or DCs alone, suggesting a role for either cross-presentation or cross-dressing (145). In response to phagocytic uptake of pathogens such as mycobacteria, salmonella, or toxoplasma, macrophages increase the release of EVs that are apparently formed as exosomes within phagosomes (146, 147). These EVs carry MHC-I, MHC-II, and costimulatory molecules such as CD86 and have the capacity to prime naive T cells (147, 148). EVs isolated from macrophages infected with pathogens stimulated CD4 and CD8 T cell responses in vitro, particularly in the presence of DCs, as well as in vivo (147–149).

Antigen presentation by extracellular vesicles from intestinal epithelial cells

Although intestinal epithelial cells express MHC-I and MHC-II, the basement membrane impedesdirect contact with T cells. However, basolaterally released pMHC-carrying EVs can overcome this barrier. Cultured intestinal epithelial cells released MHC-II-carrying EVs that could efficiently activate cognate CD4 T cells when cross-dressed onto DCs (150, 151). The A33 antigen is specifically expressed by intestinal epithelial cells but could also be detected in EVs from these cells and in murine mesenteric lymph nodes, suggesting a role for EV transfer to lymphoid tissues (150). Microscopy revealed high concentrations of MHC-II and A33 antigen within MVEs of intestinal epithelial cells, suggesting that EVs from these cells are exosomes (152). EVs that were isolated from the serum of antigen-fed mice could transfer antigen-specific CD4 T cell tolerance in syngeneic recipient mice, depending on MHC-II expression in intestinal epithelial cells, providing in vivo evidence for a role of MHC-II/antigen presentation by EVs from these cells in generating oral tolerance (153). Similarly, EVs isolated from bronchoalveolar fluid of mice that were intranasally inoculated with antigen prevented allergic reactions in recipient mice (154).

T-cell derived extracellular vesicles

T cells, like the professional APCs they interact with, release EVs with heterogeneous characteristics(6, 155). CD4 T cells that are activated via their TCRs release microvesicles from their plasma membrane into immune synapses in a TSG101- and VPS4-dependent manner (37, 155), indicating an active and specific process. T cell–derived EVs can be targeted to different types of immune cells and modify their function. In the absence of a synapse, EVs were isolated from antibody- and IL-2 -stimulated T cell cultures, and these stimulated in vitro proliferation of autologous resting CD8 T cells (156). On the other hand, EVs from activated T cells carried bioactive membrane-associated FasL and TNF-related apoptosis-inducing ligand (TRAIL), and these EVs have been proposed to help prevent potential autoimmune damage by eliminating activated T cells after a cellular immune response has taken place (157, 158). Apparently, two populations of EVs are released by DC-activated CD4 T cells, with the first (presumably microvesicles) being transferred to interacting DCs before T cell activation and the second (presumably exosomes) being transferred in response to T cell activation (159). While the first group might facilitate a productive DC–T cell interaction, the second group could play an important immunomodulatory role in intraclonal competition of T cells (159). Similarly, CD4 T cell–released exosomes were found to inhibit CTL responses and antitumor immunity (160). Yet, others observed that T cell–derived EVs could activate cognate B cells (37, 155), possibly through transfer of miRNA (5). Delivery of CD4 T cell help to B cells is dependent on cognate MHC-II/antigen complexes and CD40 signaling by the B

cells. When Th1 cells recognize antigen-presenting B cells, CD40L is upregulated on the T cells to interact with and activate CD40 on the B cells. In a recent study it was found that CD40L is transferred to and endocytosed in association with CD40 by the B cell (161). In this way, EV-mediated transfer of CD40L from T cells to B cells may sustain signaling cues that promote B cell proliferation and differentiation without the requirement of continued intercellular contact.

Potential of dendritic cell-derived extracellular vesicles in immunotherapy against cancer

Given their central role as initiator and regulator of the adaptive immune response, DCs are essential for T cell–mediated cancer immunity. Hence, many efforts are being made to develop DC-based vaccination therapies against cancer, but so far with only limited success (reviewed in 162). Current vaccination strategies are mostly based on injection of peptide, protein, or nucleic acids that can be captured by DCs in vivo. DCs have also been isolated from patients, expanded, activated, and loaded ex vivo with tumor-associated antigen, and then reintroduced into patients.

Although such approaches often resulted in the expansion of circulating tumor-specific CD4 and CD8 T cells, clinical efficacy remained low (reviewed in 163). One major problem is imposed by counteracting immune-suppressive signals from the tumor, for example, by the presence of preexisting regulatory T cells or myeloid-derived suppressor cells. As an alternative, DC-derived EVs have been developed toward a clinical grade product and tested in phase 1 and phase 2 clinical trials for their efficacy in targeting cancer. In two initial studies, tumor antigen–loaded EVs from cultured autologous immature DCs were used as vaccine (164, 165). Minimal antigen-specific T cell responses were observed in only one of these studies (165), while both reported stability of disease in some of the immunized patients, a proposed consequence of the stimulation of natural killer cells. In a more recent clinical trial, tumor antigen–loaded EVs were derived from IFN-γ- stimulated DCs, but these also could not induce strong adaptive immune responses (166). One possible limitation is that the EVs used in these clinical trials were loaded with selected peptides and therefore could be expected to activate T cells with a very limited repertoire in antigen specificity.

In mice, CD8 T cell responses improved on immunization with EVs from protein-loaded, as compared to peptide-loaded, DCs (83). Interestingly, in this setting, CTL responses were also dependent on CD4 T cells as well as on complement activation and antigen shuttling by B cells.

It has been proposed that antigen-specific B cells may recruit antigen-loaded DC–derived EVs to facilitate their transfer to FDCs. EVs isolated from cultured DCs indeed carried complement (82) and thus may be recruited by complement receptor CD21 on FDCs.

In conclusion, to be effective in future applications in cancer immunotherapy, DC-derived EVs should preferably contain costimulatory molecules as well as relevant tumor-specific antigens loaded onto both MHC-I and MHC-II. This idea is consistent with awareness that DNA and RNA vaccines, intact proteins, and synthetic long peptides that deliver antigen to both MHC-I and MHC-II are more effective in cancer immunotherapy than short-peptide antigens that are restricted to MHC-I (reviewed in (74)). A potential caveat is that in vitro–generated EVs that are used for cancer immunotherapy should preferably be produced by 100% pure immune-stimulatory DCs. Indeed, prior activation of the EV-producing DCs improved the antitumor immunity elicited by the EVs in a murine model of melanoma (167). Conversely, EVs from immature DCs can display immune-tolerizing capacities (98, 99, 101–103, 113). Primary human DCs are difficult to isolate to 100% homogeneity, and their activation is often asynchronous and incomplete. Isolated immunostimulatory EVs can therefore easily be contaminated with tolerogenic EVs, with the latter worsening the outcome of cancer immunotherapy. Furthermore, large-scale production of autologous moDCs is laborious and costly. To circumvent these problems, artificial EVs have been generated that consist of liposomes coated with pMHC-I complexes and a range of ligands for adhesion, early activation, late activation, and survival TCRs (168). In vitro, these artificial exosomes specifically triggered human antigen-specific CD8 T cells, but only in the presence of APCs, in a similar manner as natural DC-derived EVs do. As a future perspective, a homogeneous population of artificial EVs, loaded with tumor-specific pMHC-I and pMHC-II complexes and equipped with immune-activating costimulatory molecules and integrins, may prove to be effective in cancer immunotherapy. In situ, activated DCs mature and travel to lymph nodes or the spleen, where they should release their EVs, to optimize recruitment by resident DCs (28). Therefore, the activity of the therapeutic EVs is likely to be influenced by the site of administration. While systemic administration might result in clearance of EVs by macrophages and hepatic Kupffer cells (13), direct administration into the lymphatic system should enhance proper delivery and functionality (169).

Future perspectives

The immune system is very complex given the vast number of distinct cell types involved, and novel subtypes of each are being discovered at a fast pace. For example, many distinct subsets of DCs with different functional specializations in regulating immunity

have been described. Realization that all these immune cells can exchange membrane proteins, cytosolic proteins, lipids, and nucleic acids (including miRNA) by means of EVs has complicated the picture even further. First, transfer of what were previously thought to be unique marker proteins complicates the analysis and identification of specific cell types. Second, transfer by EVs of signaling molecules, such as kinases, signaling lipids, and miRNA, affects the behavior and characteristics of the receiving cells. Many immunologists were or still are wary of accepting a role for EVs in immune regulation, as this phenomenon would complicate research on the role of different immune cells in immunity even further. However, overwhelming and ever-increasing evidence for significant functions of EVs in immune regulation has been gathered over the last two decades and can no longer be neglected. Likely only a small fraction of the functions of EVs in immune regulation has been discovered. Further progress in this area would be boosted by the development of additional molecular tools for in vitro and in vivo intervention of EV release or incorporation of specific cargo therein.

Disclosure statement

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

Acknowledgments

We apologize to all authors whose work was not cited or discussed in depth because of the length limitation. This work has been supported by project number 022.004.018a, which is financed by the Netherlands Organisation for Scientific Research (NWO).

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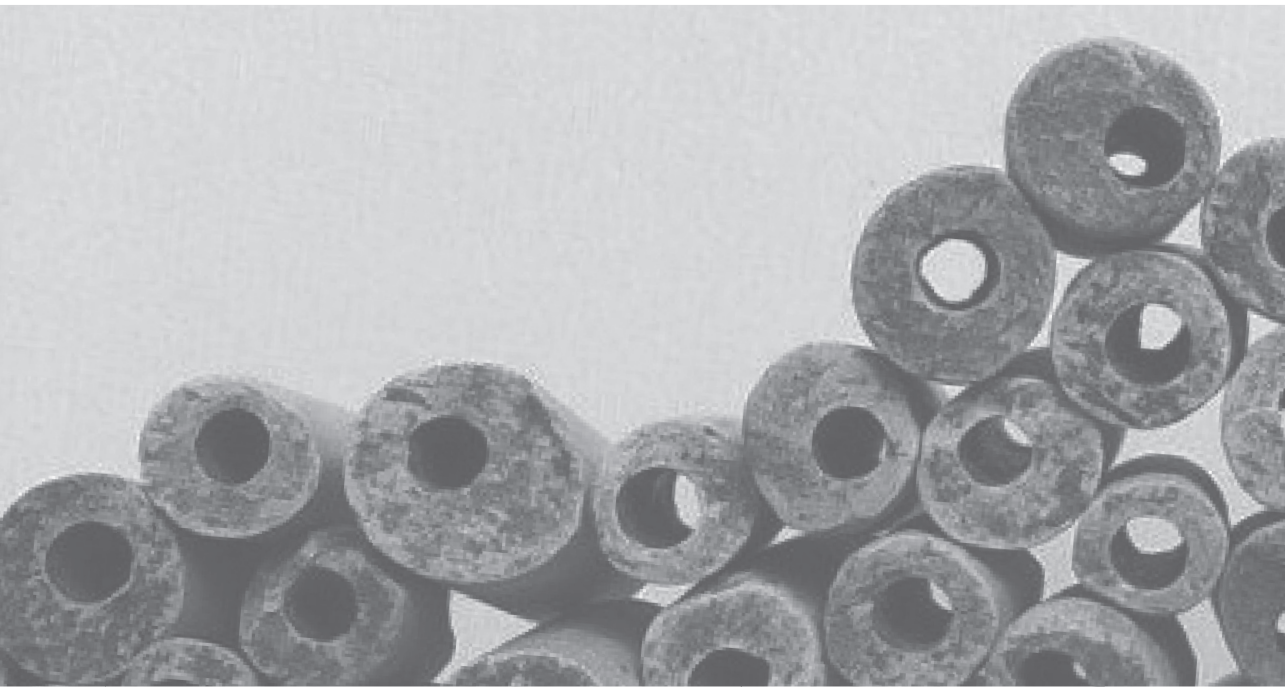

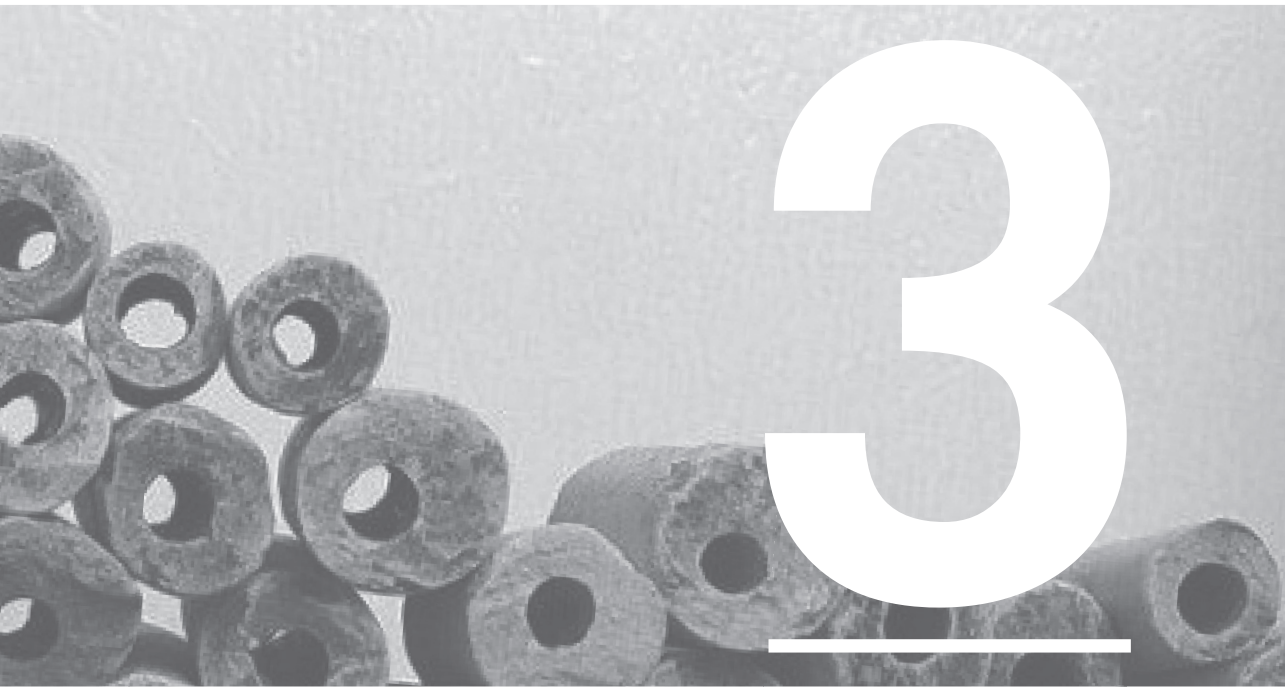

3

Bystander T-cells support clonal activation by controlling the release of dendritic-cell derived immune-stimulatory vesicles

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Frontiers in Immunology. March 2019. 10. Article 448.

Abstract

Extracellular vesicles (EV) that are released by immune cells are studied intensively for their functions in immune regulation and are scrutinized for their potential in human immunotherapy, for example against cancer. In our search for signals that stimulate the release of functional EV by dendritic cells we observed that LPS-activated human monocyte-derived dendritic cells (moDC) changed their morphological characteristics upon contact with non-cognate activated bystander T-cells, while non-activated bystander T-cells had no effect. Exposure to activated bystander T-cells also stimulated the release of EV-associated proteins by moDC, particularly CD63 and ICAM-1, although the extent of stimulation varied between individual donors. Stimulation of moDC with activated bystander T-cells also increased the release of EV-associated miR155, which is a known central modulator of T-cell responses. Functionally, we observed that EV from moDC that were licenced by activated bystander T-cells exhibited a capacity for antigen-specific T-cell activation. Taken together, these results suggest that non-cognate interactions between DC and bystander T-cells modulates third party antigen-specific T-cell responses via EV.

Keywords: *dendritic cells, T-cells, extracellular vesicles/exosomes, HLA class I, antigen presentation*

1 Introduction

Dendritic cells (DC) are sentinel cells that survey the microenvironment of barrier tissues such as the skin or mucosae for the presence of danger-associated molecules and pathogens. DC initiate adaptive immune responses by presenting peptides from exogenously acquired antigens onto Major Histocompatibility Complex (MHC) class I molecules to CD8+ T-cells (cross-presentation), or onto MHC class II molecules to CD4+ T-cells (Savina and Amigorena 2007). Upon sensing pathogen-derived molecules, such as lipopolysaccharide (LPS), DC mature into potent antigen presenting cells (APCs) that mobilize towards lymphoid tissues where they can encounter and stimulate rare antigen-specific naive B- and T-cells. When antigen-specific CD4+ T-cells are stimulated by cognate MHC class II-peptide complexes on activated DC, they increase their CD40 ligand (CD40L) expression, which in return can license DC to mature further into an APC phenotype that can prime the development of antigen-specific CTL (Schoenberger et al. 1998; Flinsenberg et al. 2015). Activated CD4+ T-cells can, however, also induce costimulatory DC in the absence of any innate DC priming, affecting all DC in the microenvironment, including those that lack specific antigen (Spörri and Reis e Sousa 2003). Similarly, CD8+ T-cells can also induce DC maturation independently of CD40, in absence of innate stimuli (Ruedl, Kopf, and Bachmann 1999; Mailliard et al. 2002). Thus, DC phenotype and efficiency is shaped by the combined actions of innate and adaptive signals. It is not well understood how exactly bystander T-cells improve the efficiency with which DC activate cognate T-cells. To further elucidate this process, we set out to investigate the effect of bystander T-cells on the release of extracellular vesicles (EV) by DC. DC secrete antigen presenting EV which, depending on the status of the originating DC, can have either tolerogenic or immune-stimulatory effects when interacting with T-cells (Lindenbergh and Stoorvogel 2018; Robbins and Morelli 2014). The capacity of EV to present antigen depends on the maturation status of the parental DC (Chaput et al. 2004), and the presence of costimulatory molecules on the EV (E Segura et al. 2005). We report here that incubation with activated bystander T-cells changed the phenotype of LPS-experienced DC, and modulated their EV-release. Functionally, EV that were released by DC upon contact with activated bystander T-cells elicited stronger peptide-specific T-cell responses than did EV induced by non-activated bystander T-cells. We propose a role for DC-derived EV in inflammatory conditions, where interactions between matured DC and activated bystander T-cells stimulate the release of EV by DC, to support subsequent antigen-specific T-cell activation.

2 Materials & Methods

2.1 Cell culture and processing

Peripheral blood mononuclear cells (PBMCs) from healthy donors were isolated from lithium heparinized blood samples using Ficoll isopaque density gradient centrifugation (GE Healthcare). CD14+ monocytes were enriched using positive selection with CD14+ MicroBeads (Miltenyi Biotec). CD14+ cells were cultured in 6-wells plates (Nunclon, Thermo Scientific) at a concentration of 1 to 1,5 x 10^6/mL at 37°C and 5% CO_2 for five days in RPMI 1640 GlutaMAX (Gibco), 1% Penicillin/Streptomycin (Gibco) and 20% heat inactivated, 0,2 µM filtered FCS (Biowest) supplemented with 450 U/mL rhGM-CSF (Immunotools) and 300 U/mL IL-4 (Immunotools). Cytokines were replenished after three days. Purity of CD14+ cells was determined using flow cytometry, and only cultures containing ≥90% CD14+ cells were used.

T-cells were isolated from the CD14-depleted fraction of PBMCs (from healthy donors) by negative selection using the Pan T Cell Isolation kit (Miltenyi Biotec). Isolated T-cells were cultured in RPMI 1640 (Gibco) containing 1% Penicillin/Streptomycin (Gibco), 1% L-glutamin (Gibco) and 10% heat inactivated, 0,2 µM filtered FCS (Biowest). T-cells were activated with 5 ng/mL phorbol 12-myristate 13-acetate (PMA) (Sigma) and 1µg/mL Ionomycin (Sigma), or alternatively, when indicated, by Dynabeads coated with anti-CD3 and anti-CD28 (Gibco). After 16 hours, cells were harvested and, if applied, magnetic Dynabeads were removed. Non-activated and activated T-cells were mildly fixed with 0.4% paraformaldehyde (PFA) (Electron Microscopy Sciences) in PBS for 30 seconds. The fixative was quenched with 0.2M glycine, and T-cells were washed by centrifugation, twice with cold PBS (Gibco) and once with culture medium. After washing, T-cells were resuspended in EV-free moDC medium and stored at 4°C. EV-free medium was prepared by ultracentrifugation for 18 hours (100,000 x g at 4°C). When indicated, moDC were activated by culturing for four hours in the presence of 100ng/mL ultrapure LPS (*E. coli* strain O111:B4, Invivogen). Activated moDC were washed by centrifugation to remove LPS and resuspended in EV-free medium. Subsequently, fixed T-cells were added 1:1 to the activated moDC. For microscopy purposes, T-cells were co-cultured with moDC either in direct contact or in separation by transwell inserts with a pore size of 1.0µm (Greiner bio-one). After 18 hours, ice cold PBS was added in excess, and cells and culture supernatant were harvested. Blood from healthy volunteers was obtained following institutional ethical approval (www.umcutrecht.nl/METC), METC protocolnumber 07-125/C. The experiments abide by the Declaration of Helsinki principles for human research ethics.

2.2 EV isolation, protein deglycosylation and Western blotting analysis

EV were collected from culture media by differential (ultra)centrifugation at 4°C, as published (G Raposo et al. 1996). Briefly: cells were removed by centrifugation twice for 10 minutes at 200 x g, followed by two times 10 minutes at 500 x g at 4°C. Next, the samples were centrifuged sequentially at 10,000 x g (30 min, 8900 rpm, 4°C) and at 100,000 x g (65 min, 28,000 rpm, 4°C) in polyallomer tubes (Beckman Coulter) using a swing-out rotor (SW-40, Beckman Coulter). For antigen presentation assays, 100,000 x g pellets were resuspended in EV-free culture medium and stored at 4°C. For Western blotting analysis, 100,000 x g pellets were lysed in non-reducing SDS-PAGE sample buffer.

For deglycosylation assays, cell suspensions were lysed in Triton X-100 buffer with complete protease inhibitor mix (Roche). Subsequently, the lysate was spun at 12.000 rpm, and the supernatant was heated to 100°C for 10 minutes followed by overnight deglycosylation at 37°C using either EndoH or PNGase F (New England Biolabs) in presence of their respective glycoprotein buffers. After deglycosylation, 4x SDS-PAGE sample buffer was added to the samples.

For Western blotting, proteins were separated by 10% SDS-PAGE and transferred to 0.45 µm polyvinylidene difluoride (PVDF) membrane (Merck Millipore). The blots were blocked and incubated with antibodies in PBS containing 0.2% gelatin from cold water fish (Sigma) and 0.1% Tween-20. Immunodetection was performed using mouse anti-human CD9 (clone HI9a; 1:2000; Biolegend), mouse anti-human CD63 (clone TS63; 1:2000; Abcam), mouse anti-human CD81 (clone B-11; 1:400; Santa Cruz), or mouse anti-human HLA-B,C (some A) (clone HC-10; 1:400; kindly provided by E.J.H.J. Wiertz), followed by HRP-conjugated goat anti-mouse IgG and IgM (1:10,000; Jackson). HRP activity was detected using ECL (SuperSignal West Dura Extended Duration Substrate, Thermo Scientific) and a ChemiDoc MP Imaging System (BioRad). Relative intensity data were analyzed using Image Lab V5.1 (BioRad).

2.3 Microscopy

For differential interference microscopy (DIC), moDC and T-cells were mixed at concentrations of 1,25 x 10^5 cells/mL each and co-cultured overnight as indicated above. Imaging was performed using a Leica DM IRBE microscope with LMC40 and 40x objective combined with a Leica D-LUX 3 (LMS) camera. For confocal microscopy, moDC and T-cells were seeded on glass coverslips, each at 2x10^5 cells/mL. After overnight incubation, cells were fixed for 30 min with 4% paraformaldehyde in 0.1M Phosphate buffer at pH 7.4, followed by quenching and permeabilization in PBS containing

20mM NH_4Cl, 2% BSA (Sigma) and 0.1% w/v saponin (Sigma). Subsequent labelling and washing was performed in PBS containing 2% BSA and 0.1% saponin. HLA class II was labelled with CR3/43 (1ug/mL, DAKO) for 45 minutes, followed by Alexa-488 labeled goat anti-mouse IgG (1 µg/mL, Invitrogen) for 30 minutes. Nuclei were labelled with DAPI (4',6-diamidino-2-phenylindole, 23,8 µM, Thermo Fisher Scientific) for one minute. Labelled coverslips were finally washed with water and embedded in Prolong Diamond (Thermo Fisher Scientific). Images were acquired using a NIKON A1R confocal microscope with 40x Plan Apo objective (NA 1.3), with regular lasers and filter settings to detect DAPI and Alexa488. Overviews of the cultures were generated by scanning 7x7 image fields at 3 positions in the Z axis at 1.5 µm steps. Representative regions of 300x300 pixels were selected and processed in NIS elements 5.02 (Nikon Microsystems, Europe). Fluorescence images were captured with identical settings, and maximum intensity projection was performed.

2.4 RNA isolation and qPCR

Small RNA was isolated from EV pellets using the miRNeasy micro kit according to manufacturer's instructions (Qiagen). The RNA yield and size profile were analyzed using the Agilent 2100 Bioanalyzer with Pico 6000 RNA chips (Agilent Technologies). cDNA was generated from 3uL EV-derived small RNA using the miScript RT2 kit (Qiagen). At least 20 pg RNA was used per qPCR reaction, and mixed in 8µL reactions in triplicate with 100nM primers (IDT, see table) and 4 µL SYBR Green Sensimix (Bioline). Control samples without RT-enzyme were used to confirm absence of genomic DNA and non-specific amplification. Cycling conditions were 95°C for 10 minutes, followed by 50 cycles of 95°C for 10 seconds, 57°C for 30 seconds and 72°C for 20 seconds. All PCR reactions were performed using the Bio-Rad iQ5 Multicolor Real-Time PCR Detection System. Quantification cycle (Cq) values were determined using Bio-Rad CFX software with automatic baseline settings, averages of triplicates were used for further analysis. Thresholds were set in the linear phase of the amplification curve. Forward primer sequences for miRNA targets were designed based on sequence information from miRBase, the U6 primer sequence was taken from Galiveti et al (Galiveti et al. 2010) (**Table 1**). As a reverse primer, the miScript universal reverse primer was used in all reactions.

Table 1

RNA species	Primer sequence from 5' to 3'
hsa-miR-30b-5p	TGTAAACATCCTACACTCAGCT
hsa-miR-155-5p	TTAATGCTAATCGTGATAGGGGT
hsa-miR-146a-5p	TGAGAACTGAATTCCATGGGT
U6-F	CTCGCTTCGGCAGCACA

2.5 Preparation of NLV-expanded T-cells and NLV specific T-cell activation assay

For antigen-specific T-cell activation experiments we exclusively used moDC and bystander T-cells from HLA-A*02.01 positive donors, as determined by flow cytometry. CD8 T-cells recognizing the HCMV (human cytomegalovirus) pp65-derived peptide (NLV) were isolated and expanded as described (Flinsenberg et al. 2012) with minor modifications. In brief, T-cells from an HLA-A*A02.01 positive donor were cultured with THP-1 cells presenting NLVPMVATV peptide (Proimmune). After one week, T-cells positive for HLA-A2/$pp65_{495-503}$ tetramers were sorted and incubated with irradiated, T-cell depleted (using positive CD3+ sort from Miltenyi Biotec), PBMCs ($1x10^5$ cells/mL, irradiated with 70 Gy) from three healthy donors. The cells were cultured in the presence of 1µg/mL leucoagglutinin PHA-L (Sigma-Aldrich) and 120 U/mL of recombinant human IL-2 (Immunotools) and re-stimulated and expanded during several culturing cycles before freezing in aliquots. Aliquots of expanded T-cells were thawed for each experiment, and incubated in medium containing 0.1% Monensin/Golgistop (BD). Portions of the EV isolates were pre-incubated for one hour with 10^{-7} M NLVPMVATV peptide (NLV) prior to their addition to the T-cells. EV isolated from 200.000 moDC were added to 50.000 T-cells in a total volume of 50 µL in a round-bottom 96-wells plate (Thermo Scientific), and incubated for 4 to 5 hours before assaying by flow cytometry intracellular cytokines and cell surface marker expression.

2.6 Flow cytometry

Cells were blocked in flow cytometry buffer (PBS with 0.5% BSA and 0.02% NaN_3) supplemented with 0.5% NMS (Fitzgerald 88R-M002). For intracellular staining, cells were permeabilized using cytofix/perm buffer (BD) and washed in perm/wash buffer (BD). CD3 was detected using Pacific Blue (PB)-labeled mouse-anti human CD3 (clone UCHT-1; 1:50; Beckman Coulter), CD8 with Fluorescein (FITC)-labeled mouse anti-human CD8 (clone RPA-T8; 1:25; BD), CD107a (LAMP-1) with Phycoerithrin (PE)-labeled mouse anti-human CD107a (clone H4A3; 1:25; BD), IFNγ with PE-Cy7-labeled mouse anti-human IFNγ (clone 4S.B3; 1:200; BD), TNFα with Allophycocyanin (APC)-labeled mouse anti-human TNFα (clone mab11; 1:200; Sony Biotech), HLA-A2 with PE-labeled

mouse anti-human HLA-A2 (clone BB7.2; 1:100; Biolegend), and CD69 with FITC-labeled mouse anti-human CD69 (clone FN50; 1:50; Miltenyi Biotec). PE-labeled murine IgG1,k (clone MOPC-21; BD) and FITC-labeled murine IgG1,k (clone MOPC-21; BD) were used as isotype controls. Data were collected on a FacsCanto II (BD) flow cytometer and analyzed with FlowJo 10.0 software (Treestar).

2.7 Statistical analysis

Significance of differences was determined by two-sided paired t-tests using GraphPadPrism 7 software. When indicated, we used a Wilcoxon's signed rank test to demonstrate consistent differences between paired observations. P values <0.05 were considered statistically significant.

3 Results

3.1 Response of activated moDC to activated bystander T-cells.

Breaching of epithelia that cover barrier tissues elicits antigen-specific immune activation, which relies on the traffic of maturing tissue-derived DC to deliver antigen-specific cues to secondary lymphatic organs. Here, such DC interact most frequently with non-cognate bystander T-cells, some of which might provide further maturation signals to the DC. In our search for signals from bystander T-cells to maturing DC, we first examined the effects of bystander T-cells on LPS activated autologous moDC (**Figure 1A**). To eliminate any possible contribution of EV from bystander T-cells, non-activated or T-cells that had been activated with PMA and Ionomycin were mildly fixed with PFA prior to addition to moDC (Bertho et al. 2003). Fixation did not change the exposed activation markers on the T-cells (**Figure S1A**). Addition of LPS alone to moDC (control) resulted in the outgrowth of dendrites, characteristic for DC maturation (**Figure 1B:i**). Subsequent exposure to non-activated bystander T-cells did not induce further morphological features of maturation (**Figure 1B:ii**). In contrast, exposure to activated bystander T-cells triggered LPS-experienced moDC to form large clusters of stretched cells, which firmly adhered to the culture dish (**Figure 1B:iii**). This effect was not caused by the fixation of the bystander T-cells, as moDC incubated with bystander T-cells that had not been chemically fixed showed the same characteristics (**Figure S1B**). Furthermore, the same DC phenotype was observed when the DC were incubated with T-cells that were activated with anti-CD3/CD28 coated Dynabeads, indicating independence of the method applied for T-cell activation (**Figure S1C:i-ii**). We were unable to dissociate individual moDC from these induced clusters of cells, precluding flow cytometric analyses (our unpublished observations). Instead, we resorted to confocal microscopy to visualize the expression and distribution of human leukocyte

antigen (HLA) class II complexes. Non-activated bystander T-cells did not modulate the expression or distribution of HLA class II in already matured LPS-experienced moDC (**Fig 1C:i-ii**). Activated bystander cells, however, upregulated HLA class II expression (visualized as an increased intensity of fluorescence), both intracellularly and at the plasma membrane (**Fig 1C:iii)**. When activated T-cells were separated from the DC by using a transwell insert expression of HLA II was not increased (**Figure S1C:iii compared to S1C:ii**), indicating a requirement for direct contact of DC with activated T-cells. In conclusion, interactions with autologous activated bystander T-cells resulted in phenotypic maturation of moDC, whereas encounters with non-activated bystander cells did not.

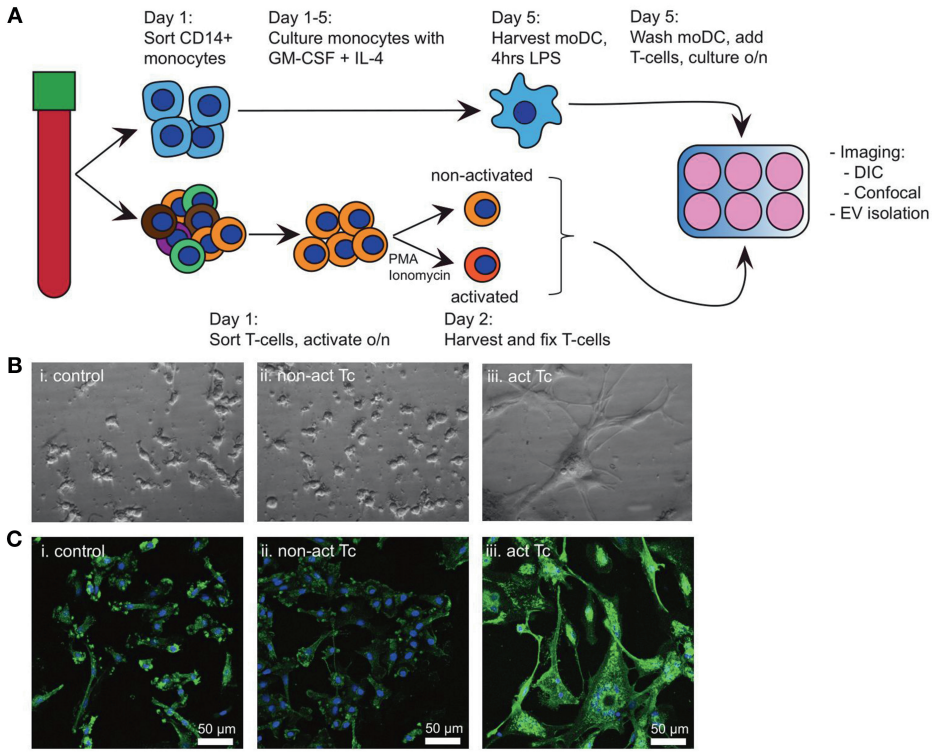

Figure 1: Activated T-cells alter the phenotype of LPS-experienced moDC
(A), Schematic overview of the experimental design. Monocytes and T-cells were sorted separately from the same PBMC-isolate. A proportion of the T-cells (bystander T-cells) was activated o/n with PMA and Ionomycin, after which non-activated and activated T-cells were mildly fixed in 0.4% PFA. Meanwhile, moDC were differentiated from monocytes during five days culturing in the presence of GM-CSF and IL-4, and subsequently activated for four hours with LPS. Subsequently, LPS-experienced moDC and bystander T-cells were incubated o/n in a 1:1 ratio.

Figure 1: Continued

(B), DIC images of o/n cultured moDC. **i**, control (LPS-matured only); **ii**, no effect of fixed, non-activated T-cells; **iii**, stretching and clustering of moDC in the presence of fixed, activated bystander T-cells. Representative images from one out of three independent experiments are shown. All images were acquired using the same magnification. **(C)**, Fluorescence microscopy detection of HLA class II on moDC (green), and nuclei (blue) in samples similar to (B). Representative images from one out of three independent experiments are shown; **i**, control (LPS-matured only); **ii**, no effect of non-activated T-cells on moDC phenotype; **iii**, moDC stretch, cluster, and HLA II expression is increased upon incubation with activated T-cells. HLA class II expression in increased. Bars indicate 50μm.

3.2 Activated bystander T-cells induce release from moDC of EV-marker proteins and miRNA species.

We next asked whether activated bystander T-cells influenced the release of EV by moDC. As before, to ensure that we collected moDC-derived EV only, we chemically fixed the bystander T-cells prior to addition to moDC. After overnight co-incubation of moDC and fixed bystander T-cells, EV were collected from culture media by differential (ultra)centrifugation. With this procedure, large EV and cell debris were discarded by centrifugation up to 10,000 x g, and only small EV were collected during a final centrifugation step at 100,000 x g (Raposo et al. 1996). These EV were analyzed by Western blotting for the presence of EV marker tetraspanins (CD9, CD63, CD81), the antigen-presenting molecule HLA class I, and intercellular adhesion molecule ICAM-1 (**Figure 2A**). From here on we refer to EV released by moDC upon contact with non-activated bystander T-cells as “EV n.a.”. Their counterparts, released by moDC upon contact with activated bystander T-cells, are referred to as “EV act”. We found that the release of EV-associated markers by moDC remained largely unaffected by the presence of non-activated bystander T-cells (EV n.a.). In contrast, activated bystander T-cells stimulated the release of EV-associated proteins by moDC (EV act). Due to variation between donors, however, significant increases were established only for HLA-I (1.8 fold, ± 1.6, n=6, p=0.0344), CD63 (3.0 fold, ±2.3, n=5, p=0.0407) and ICAM-1 (1.9 fold, ± 1.4, n=5, p=0.0375) for the combined results from five to six independent donors (**Figure 2B**). Interestingly, incubation with activated bystander T-cells resulted in a slight decrease in the apparent molecular weight of CD63 (**Figure 2A**). Of note, this change in migration by SDS PAGE was not exclusive for the EV-associated CD63, as we also observed this for CD63 in the cell lysate of moDC (**Figure S2**). Further analysis suggests that the change in mobility is caused by differences in glycosylation of the protein (**Figure S2**).

We next asked whether encounters with bystander T-cells would influence the loading of mi-RNA-species into moDC-derived EV. We first analyzed the relative quantity of total

small RNA isolated from EV from equal numbers of moDC at different experimental conditions. Relative to the LPS only condition, we observed a significant, albeit small, increase in total small RNA in response to EV n.a. (p=0.0419) (**Figure 2C**). The increase in release of total EV associated small RNA by moDC in response to interaction with activated bystander T-cells was highly variable between donors and did not reach significance (**Figure 2C**). Next, the abundance of specific miRNA species with known immune-modulatory effects in moDC-derived EV was determined by qPCR. Because of the current lack of generally applicable reference genes to normalize RNA abundance in EV from different conditions (Mateescu et al. 2017), we quantified changes in specific miRNAs in EV isolated from equal numbers of moDC (**Figure 2D**). We found that miR-155 was increased by a Log2fold change of 3.6 (± 1.0, n=4, p=0.061) in EV act compared to EV n.a.. Additionally, we observed an increase in miR-146a and miR-30b in EV act for some of the donors, although the difference between EV act and EV n.a. did not reach significance for these miRNA species. U6, which is often used as a reference gene in EV (Stik et al. 2017), did not follow the trends observed for the different miRNAs. In conclusion, activated bystander T-cells increased the release of specific small RNAs in moDC-derived EV, such as the pro-inflammatory miR-155, which may have an effect on the immunogenic potential of these EV.

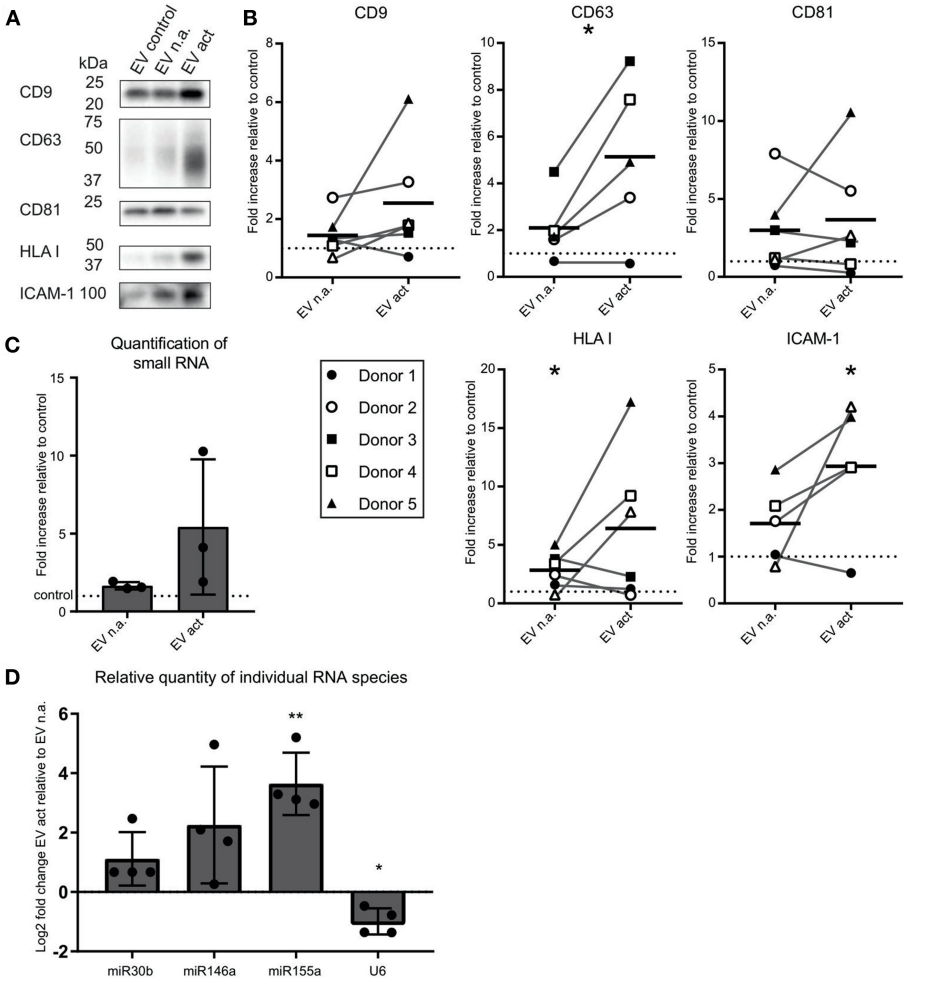

Figure 2: Activated bystander T-cells modulate the release of EV-markers by LPS-stimulated moDC

(A), Western blot detection of markers in EV collected from moDC incubated with LPS only (control), or additionally with non-activated (n.a.) or activated (act) bystander T-cells. EV were isolated from the culture supernatant of equal numbers of moDC per condition. Representative experiment out of six independent experiments is shown. **(B)**, Quantification of data as in (A) for five or six independent donors from six independent experiments. Independent experiments (donors) are each represented by individual symbols. Values are plotted relative to the signal obtained for the control condition (moDC incubated with LPS only), represented by the dotted line. Horizontal lines indicate the mean. Asterisks placed above symbols indicate a significant difference between the data represented by these symbols and the control condition (Paired t-test, $p<0.05$). Asterisks placed between the columns with symbols indicate a significant difference between the data represented by the two conditions (Paired t-test, $p<0.05$)

Figure 2: Continued

(C) Release of total small RNA in EV derived from LPS-experienced moDC is increased upon addition of bystander T-cells, especially activated bystander T-cells. Quantification of relative amounts of small RNA isolated from EV pellets from co-culture supernatant, as measured with Bioanalyzer Pico. Data are from three independent donors in three independent experiments. The control condition is represented by the dotted line. **(D)** qPCR detection of specific small RNA species in EV pellets collected from LPS-matured moDC incubated with non-activated (EV n.a.) or activated (EV act) bystander T-cells. Changes in (mi)RNA levels of EV act are depicted as log2fold-change relative to EV n.a.. U6 refers to the small nuclear snRNA species U6. Quantification of data from four independent donors in four independent experiments. Asterisks placed above columns indicate a significant difference between the data represented by the column and the control condition. Paired t-test *p<0.05, **p<0.01.

3.3 Encounter with activated bystander T-cells drives secretion of immune-stimulatory EV by DC.

Considering the reported immune-modulatory functions of the miRNA species that we found to be increased in EV act, we set out to test the potential of these EV in driving antigen-specific immune responses. Others showed that primed- but not naive T-cells, can be activated by EV directly in the absence of bystander DC (Théry et al. 2002). To test the ability of moDC-derived EV to induce antigen-specific CD8+ T-cell activation, we resorted to the use of our established system of CD8+ T-cells, which were expanded in presence of the CMV pp65-derived NLV-peptide (Flinsenberg et al. 2012). Of note, only a subpopulation of these T-cells is NLV-reactive after expansion, and these primed cells do not display activation markers until re-stimulated. LPS-experienced moDC were incubated with fixed bystander T-cells and EV isolated from the culture medium as above. Isolated EV were subsequently incubated with the NLV peptide for one hour to allow passive binding of the high affinity NLV-peptide to HLA-A2 (Flinsenberg et al. 2015)(**Figure 3A**). Next, peptide-loaded EV were incubated with NLV-expanded T-cells, after which the T-cells were probed by flow cytometry for the presence of activation markers. In absence of the relevant peptide we only saw background labelling of the surface activation marker CD107a, and intracellular activation markers TNF and IFN could not be detected (**Figure 3B, top row**). Upon incubation with NLV-peptide-loaded n.a. EV, a small proportion of the T-cells was activated (**Figure 3B, second row**). NLV-loaded EV act consistently stimulated a higher percentage of NLV-specific T-cells to express the three cognate activation markers (**Figure 3B, third row**). Incubation with NLV-peptide alone, in the absence of EV, also resulted in antigen specific activation, albeit at lower percentages compared to T-cells incubated with NLV-loaded EV act (**Figure 3B, last row**). This residual activity is likely a result of HLA-A2 expression by and presentation amongst human T-cells themselves. After analyzing six different donors, we found that consistently more T-cells were activated by EV act + NLV as compared to

EV n.a. + NLV, as determined by TNF (p=0.0313), CD107a (p=0.0313) and IFN (p=0.0313) (**Figure 3C**). In conclusion, we demonstrate that moDC increased their release of EV markers in response to interaction with activated bystander T-cells, and that these EV are functionally active in stimulating peptide-specific CD8+ T-cells.

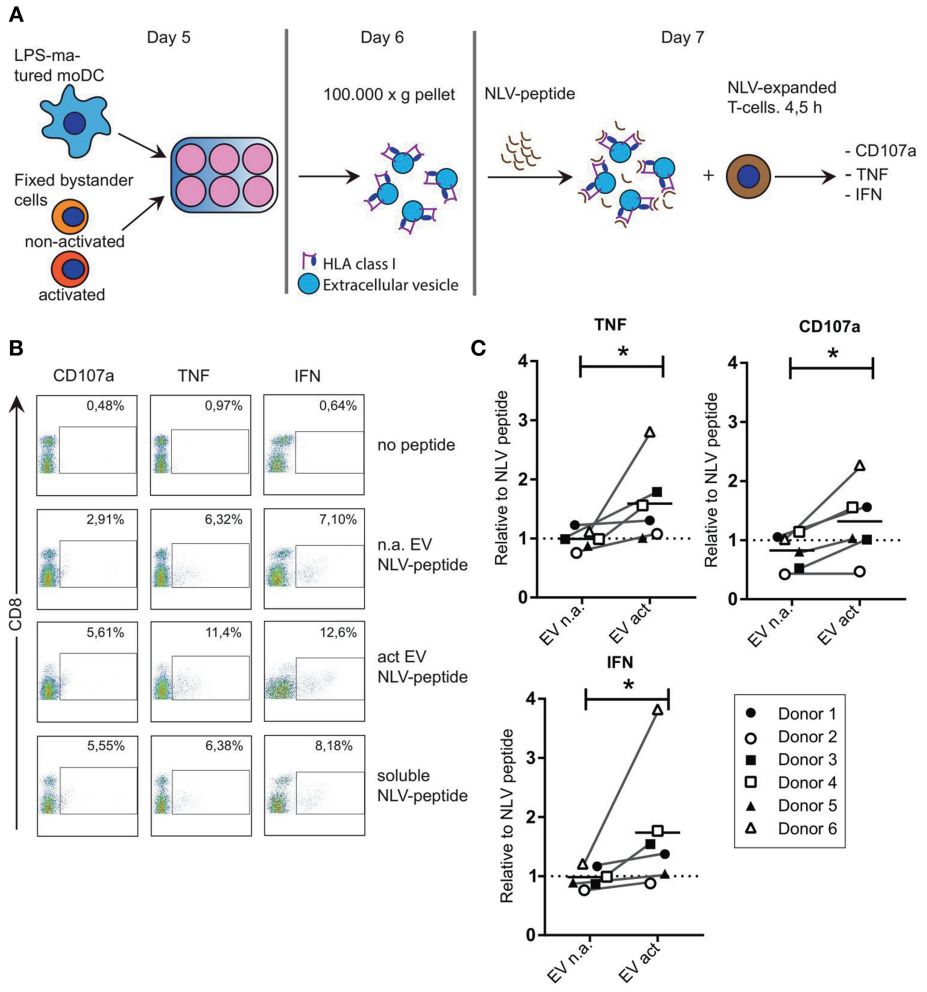

Figure 3: EV that are released from moDC in response to non-cognate interactions with T-cells can stimulate antigen specific T-cell responses.
(A), Schematic representation of the experimental workflow. LPS-experienced DC were incubated o/n with fixed, activated or non-activated bystander T-cells. The next day, EV were collected from the culture medium and incubated with NLV-peptide for one hour. Subsequently, NLV-expanded CD8+ T-cells were added to the EV and incubated for 4.5 hours, followed by flow cytometric analysis of cognate activation markers.

Figure 3: Continued

(B) Flow cytometric detection of CD8 and cognate activation markers CD107a, TNF and IFN. CD8+ T-cells were gated (not shown), after which CD8+ T-cells with increased expression of the indicated activation markers (top) were gated (boxes) and quantified as a percentage of the total number of CD8+ T-cells (top right in each plot). Representative experiment out of six independent experiments. **(C)** Quantification of T-cell activation data as in B, for six independent experiments (each represented by different symbols).-NLV-specific T-cell activation by moDC-derived EV induced by non-activated bystander T-cells (EV n.a.) or activated bystander T-cell-induced EV (EV act) are plotted, relative to the NLV-only condition (no EV present). Horizontal lines indicate the mean. All donors showed a significant increase in cognate T-cell activation in EV act compared to EV n.a.. Wilcoxon's signed rank test * $p<0.05$.

3 Discussion

DC in peripheral tissues serve as sentinels of the immune system. When DC encounter pathogens in tissues such as the lungs and intestines, they are triggered to migrate and transport antigens towards secondary lymphoid tissues to prime antigen-specific T-cell responses. During transit to lymphoid tissues, DC undergo staged maturation to support their later role in T-cell activation. Within the lymph node, different cell types influence each other's functionality. In depth knowledge on the specific interactions and their consequences is paramount for the design of effective vaccines and immune therapies. Dendritic cells are especially of interest, since they integrate signals of the innate immune system with the adaptive immune system, ultimately determining their efficiency in activating antigen-specific T-cells (Savina and Amigorena 2007). Interactions with activated bystander T-cells have been shown to increase the efficiency with which DC activate cognate third party T-cells (Spörri and Reis e Sousa 2003). This is partly mediated via upregulation of the expression of costimulatory molecules and by increased production of the T-cell stimulatory cytokine IL-12 p70 (Spörri and Reis e Sousa 2003). Interestingly, in response to maturation signals, DC also release EV with the capacity to activate cognate T-cells, either directly or via bystander cells (Zitvogel et al. 1998; André et al. 2004; Théry et al. 2002; Qazi et al. 2009). How and which stimuli most efficiently induce release of DC-derived EV that are potent in cognate T-cell activation remains largely unknown. Therefore, we set out to investigate whether bystander T-cells have a functional effect on the release of DC-derived EV with the capacity to activate cognate T-cells. To this end, we cultured LPS-experienced DC with fixed bystander T-cells, enabling us to isolate EV from DC only, to study their composition and functions. We found that LPS-experienced DC displayed a more activated phenotype upon subsequent incubation with activated bystander T-cells, as established by morphological criteria and MHC class II expression. Moreover, activated T-cells stimulated the release by DC of EV-associated ICAM-1 and CD63 as well as small

RNA species, including immune-stimulatory miR-155. Importantly, we found that EV act were significantly more efficient than EV n.a. in activating antigen-specific T-cells. Our data are consistent with earlier observations that DC-derived EV can present antigen and carry costimulatory molecules, and can thus activate primed cognate T-cells directly in the absence of activated bystander DC (Théry et al. 2002; Segura et al. 2005).

EV constitute a heterogeneous group of secreted vesicles that are variable in size and cargo, and different subclasses have been shown to be relevant in cell-to-cell communication for immune modulation (Raposo and Stoorvogel 2013; Lindenbergh and Stoorvogel 2018; Théry, Ostrowski, and Segura 2009; Robbins and Morelli 2014). The maturation status of the EV-producing DC determines the antigen presentation capacities of these EV (Segura, Amigorena, and Théry 2005). EV released by mature DC have the ability to induce cognate T-cell activation alone, or after being transferred to bystander cells (Zitvogel et al. 1998; André et al. 2004; Théry et al. 2002; Qazi et al. 2009). In a mouse model system, cognate interactions with antigen-specific primed CD4+ T-cells strongly enhanced secretion by DC of EV with the potency to present antigen, while non-cognate DC-T-cell interactions also stimulated EV release by DC, albeit to a lesser extent (Buschow et al. 2009). Our current demonstration that non-cognate interactions between activated human moDC and activated T-cells stimulated the release of antigen-presenting and/or immune-modulating EV by DC is consistent with these previous observations.

The release by moDC of EV-associated HLA I, adhesion molecule ICAM-1 and tetraspanin CD63, as well as miR-155a, was increased in response to activated bystander T-cells. The increase of these markers could be attributed either to an increase of number of EV, changes in protein and miRNA load per individual EV, or changes in composition of different types of EV within the total EV population. Given the limited change in EV marker content, as observed here, it is difficult to discriminate between these possibilities. Indeed, without further purification, precise determination of EV number and characteristics is challenging using currently available techniques (Raposo and Stoorvogel 2013). As this would require larger quantities of EV than could be isolated with the current setup, we were unable at this stage to more precisely determine dissimilarities between EV released at different experimental conditions. Another complication is that a proportion of the released EV is recruited by activated bystander moDC. Therefore, isolated EV are likely to represent only a subset of the total EV population. Furthermore, in our analysis we omitted large EV that may have been lost in the 10.000 x g pellets. Others found that particularly small EV, pelleting at 100.000 x g, promoted Th1 cytokine secretion (IFN-γ), while large EV pelleting at 10.000 x g

stimulated secretion of Th2 cytokines (Tkach et al. 2017). Given that human moDC particularly induce Th1 differentiation (Rissoan 1999), we here focused exclusively on small EV, isolated at 100.000 x g and the Th1 cytokines IFN and TNF. Taken together, it should be noted that although our quantitative assessment of EV-associated markers is indicative for either quantitative or qualitative differences in EV release, restrictions of isolation procedures and dynamic processes such as recruitment of (selective) EV populations by bystander moDC and T-cells are likely to have influenced these data.

The presence of ICAM-1 on DC-derived EV, and ICAM-1 dependent recruitment of EV by bystander DC were reported to be essential for their priming activity on naive T-cells (Segura et al. 2005). However, DC-derived EV can also be recruited by activated T-cells via LFA-1 – ICAM-1 interactions (Nolte-'t Hoen et al. 2009), and peripheral primed CD8 T-cells could be activated by isolated EV from moDC in the absence of bystander DC (Admyre et al. 2006). Our current demonstration that antigen loaded EV from bystander T-cell stimulated DC could directly activate antigen specific CD8 T-cells is consistent with these earlier observations.

EV contain different RNA molecules (Valadi et al. 2007), that upon transfer can be functional in target-cells (Alexander et al. 2015). EV-associated RNA species are intensively studied for their role in disease (Kosaka et al. 2016) and are also scrutinized for their immune-modulatory functions (Fernández-Messina, Gutiérrez-Vázquez, and Rivas-García 2016; Driedonks et al. 2018). We found an increase in the release of several EV-associated miRNA species by DC in response to activated bystander T-cells. Of the miRNA species tested, particularly miR-155 was highly enriched (approximately 15-fold) under influence of activated bystander T-cells. MiR-155 is known to stimulate both T-cell activation (O'Connell et al. 2010) and the differentiation of immune-stimulatory DC (Cubillos-Ruiz et al. 2013). Rab27a and Rab27b double-knockout mice are deficient in exosome secretion (Ostrowski et al. 2010), and found to be refractory to stimulation with LPS, indicating a in vivo role for exosomes in the response to endotoxins (Alexander et al. 2017). LPS responsiveness could be rescued by i.p. injection of EV isolated from wildtype DC, but not by EVs from miR-155 knock out DC. These authors also demonstrated that expression of SHIP1 and IRAK-M, which are regulators of LPS responses, are direct targets by EV-associated miR-155 (Alexander et al. 2017). Together with these data, our observations indicate that in EV act-associated miR-155 may be released in response to innate inflammatory conditions (e.g. LPS and abundance of activated T-cells), to support subsequent adaptive immune responses. EV-associated miR-146a-5p also appeared to increased in response to activated bystander T-cells, albeit to a lesser extent. MiR-146a is involved in regulation of Th1 responses by regulatory T-cells (Lu et al. 2010)

and is important to prevent autoimmune responses (Boldin et al. 2011). We found that miR30b-3p was not significantly increased by the presence of bystander T-cells in DC-derived EV. Consistent with this observation, miR30b-3p has been reported to be elevated in tolerogenic DC (Stumpfova et al. 2014). To be able to compare the relative quantities of miRNAs associated with immune-stimulatory functions, we compared the relative quantities of the immune-stimulatory miRNA species with the snRNA species U6, as this RNA-species is not associated with immune-regulatory effects. Consistent with this idea, U6 was slightly elevated in EV derived from DC incubated with non-activated bystander T-cells, but not in EV from DC incubated with activated bystander T-cells. Collectively, these findings suggest that incorporation of immune stimulatory miRNA species, such as miR146a and miR155, into EV derived from LPS-experienced moDC is selectively upregulated in response to activated bystander T-cells.

Based on our data, we propose a model in which pathogen-experienced DC travel towards the lymph node (**Figure 4-1)** to encounter both activated and non-activated bystander T-cells, the ratio of which is dependent on the prevailing immune-status in that specific lymph node. Interactions with activated bystander T-cells further stimulate the activation status of the DC, and induce the release of EV by the activated DC in the lymph node. These EV act are enriched for molecules that can stimulate antigen specific T-cells, including antigen presenting HLA-I loaded with pathogen-derived peptides, as well as co-stimulatory molecules, membrane organizing proteins, and immune-modulatory RNA-species (**Figure 4-2**). The EV may stimulate cognate T-cells directly, or via bystander DC, ultimately resulting in cognate T-cell activation. Thus, the pre-existing balance of activated and non-activated bystander cells in the lymph node helps shaping the immune response that is generated against the pathogen presented by the DC (**Figure 4-3**).

The promise of DC-derived EV as cancer-therapy was posed in the early stages of EV-research (Zitvogel et al. 1998). Since then, patient DC-derived EV have been tested in clinical trials for their capacity to activate T-cell responses towards clearing cancer cells, but only limited success has been reported until now (Besse et al. 2016; Escudier et al. 2005; Morse et al. 2005). One potential complication is that DC-derived EV can have tolerogenic or stimulatory capacities, and it is ill-defined which experimental conditions drive the generation of such phenotypically opposing EV. Our data, obtained with human cells, show that autologous activated bystander T-cells induce the release of DC-derived EV and endow them with immune-stimulatory capacities. These observations may help further optimization of DC-derived EV-based vaccines.

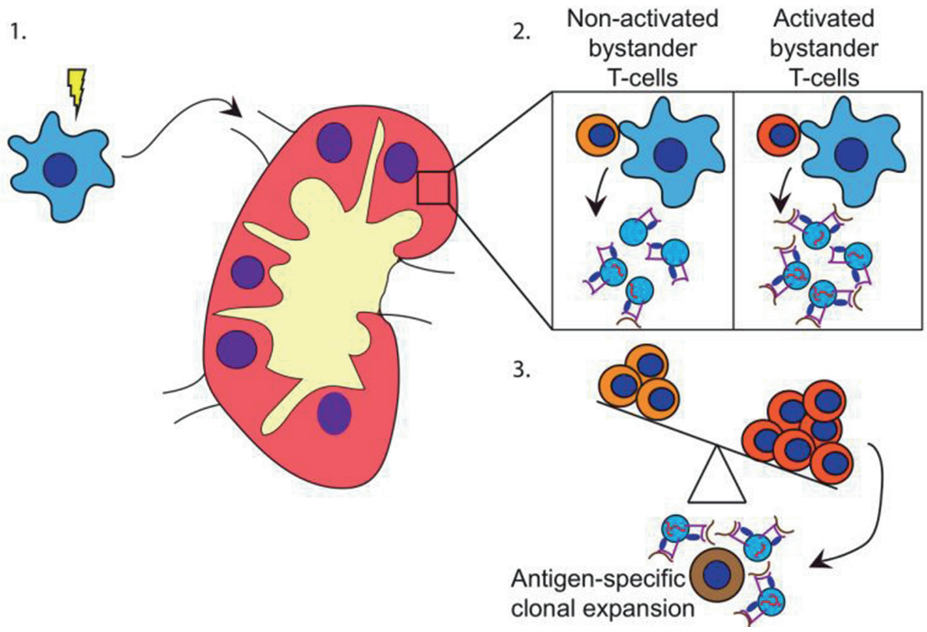

Figure 4: Hypothesis
1. DC (blue) encounter maturation stimuli and migrate towards the (draining) lymph node. **2.** In the lymph node, DC encounter both activated and non-activated bystander T-cells. Upon the interaction with activated bystander T-cells, DC release EV containing HLA class I-pathogen-derived peptide complexes and immune-stimulatory RNA-species. **3.** Depending on the ratio of activated versus non-activated bystander T-cells, the DC releases EV with a capacity to activate primed cognate T-cells. EV may also stimulate T-cells via cross dressing of bystander DC (not shown).

4 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

5 Author Contributions

ML designed and performed experiments, analysed data and wrote the manuscript. DK, EB, EV and TD performed experiments. RW supervised the design and execution of microscopy experiments. WS and MB oversaw the project, helped designing experiments and wrote the manuscript.

6 Funding

This work has been supported by project number 022.004.018a, which is financed by the Netherlands Organisation for Scientific Research (NWO), and a seed grant awarded by Utrecht University to WS and MB.

7 Acknowledgments

We would like to thank the Centre for Cell Imaging (CCI) at Utrecht University for experimental support, and NWO for financial support.

8 Data Availability Statement

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

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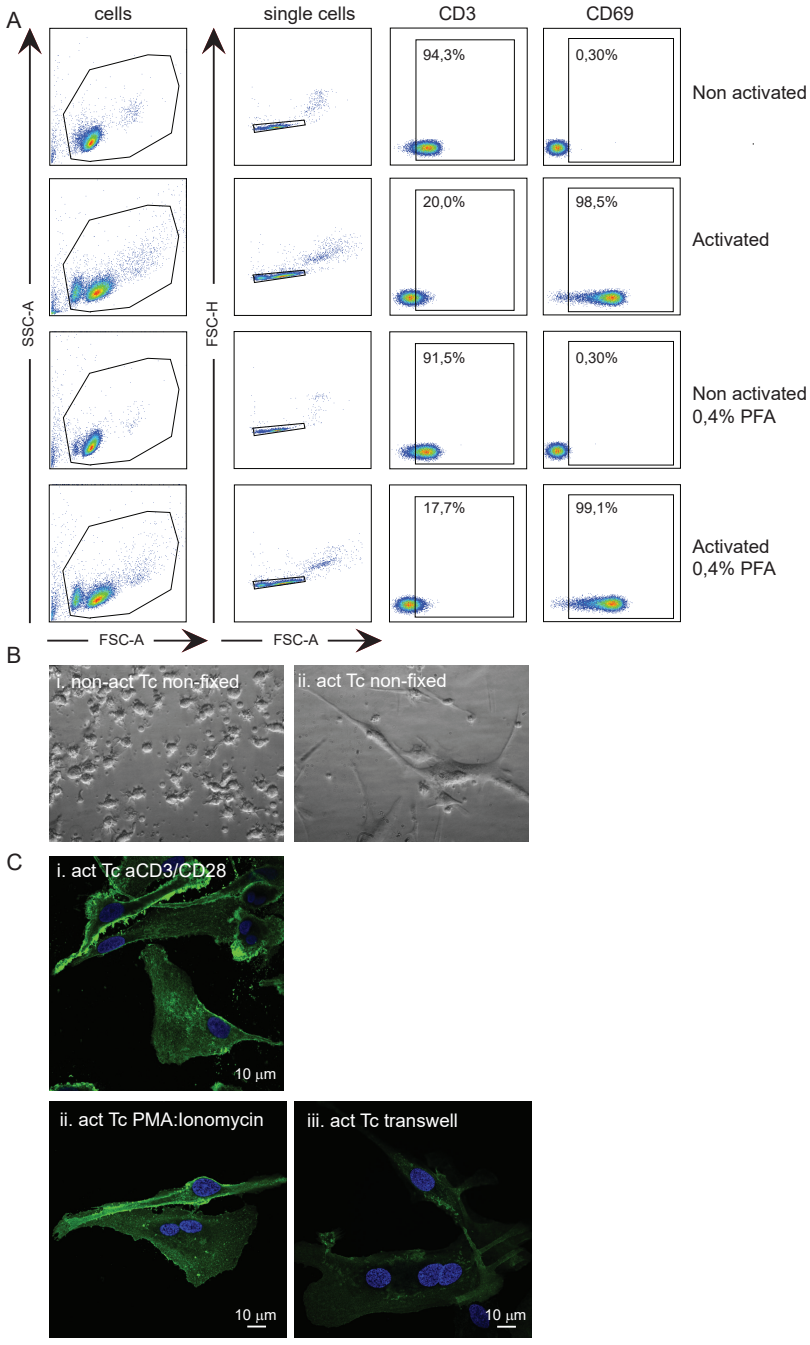
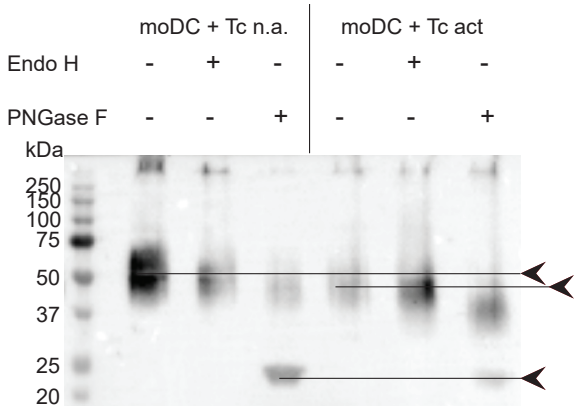
Supporting information



Supplementary figure 1: Fixation of T-cells did not affect the detection of activation markers on T-cells or T-cell-induced activation of moDC

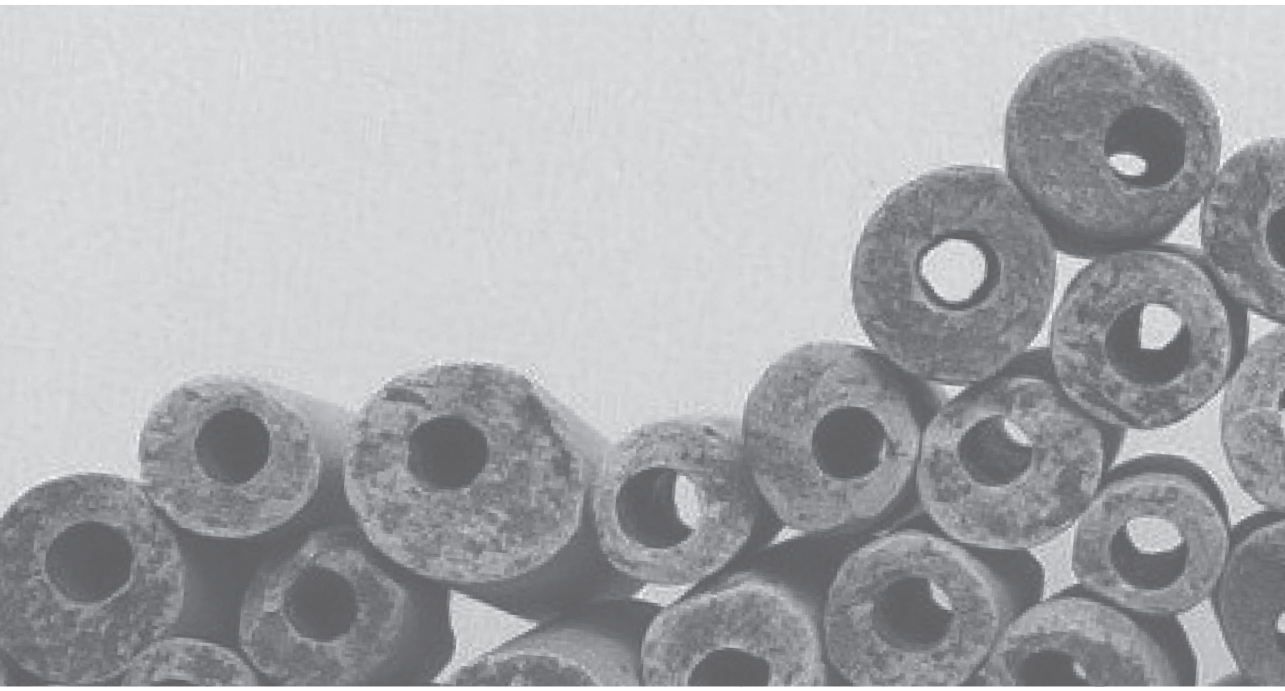
Supplementary figure 1: Continued

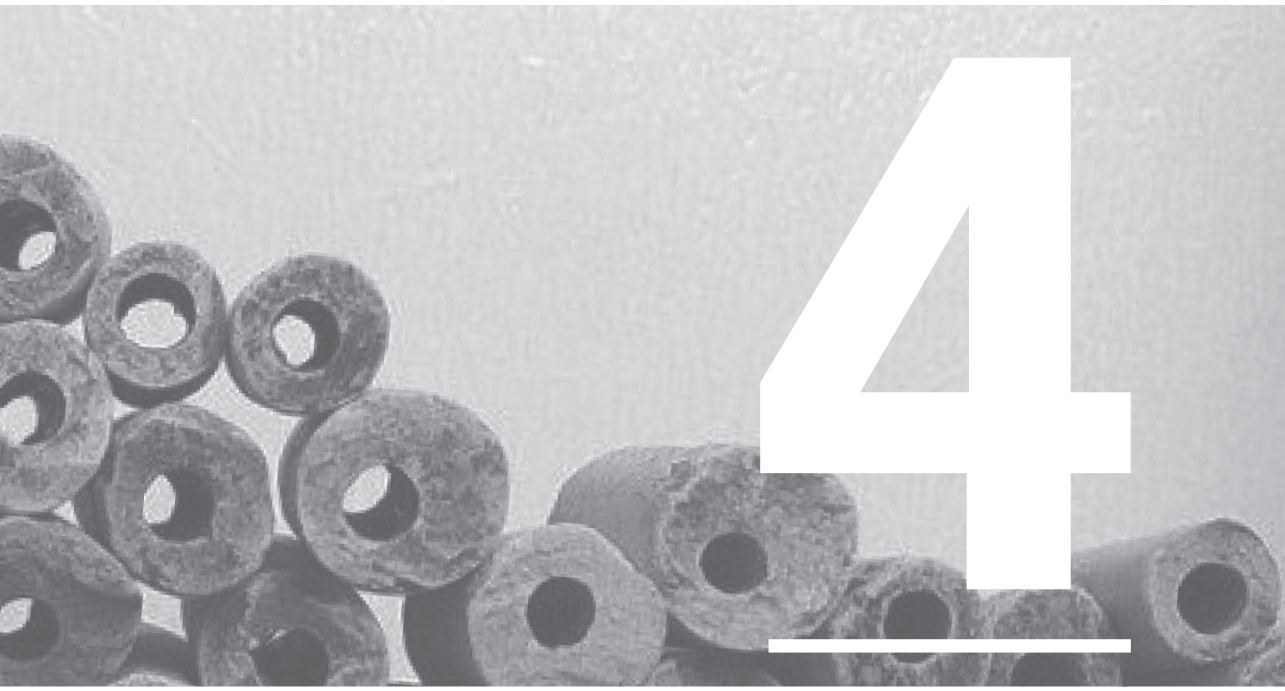
(A), The entire T-cell population was activated during o/n incubation with PMA and Ionomycin, as demonstrated by downregulation of CD3 and upregulation of CD69 surface expression. Fixation in 0,4% PFA for 30 seconds (prior to antibody labelling) did not affect detection of activation surface markers on T-cells. Data are representative of 4 independent experiments. **(B)**, Effect of non-fixed bystander T-cells on moDC. Bystander T-cells in **(i)** were not activated. In comparison, bystander T-cells in **(ii)** were activated by o/n incubation with PMA:Ionomycin. Representative DIC images from three independent experiments were acquired with the same magnification. **(C)**, Control conditions to exclude PMA:Ionomycin-induced artifacts and dependence on contact between moDC and T-cells. Fluorescence microscopy detection of HLA class II on moDC (green) and nuclei (blue). MoDC were cultured o/n in the presence of fixed T-cells. MoDC that were stimulated by anti-CD3/CD28 stimulated T-cells **(i)** revealed the same characteristics as moDC that were stimulated with PMA:Ionomycin activated T-cells **(ii)**. MoDC that were separated from PMA:Ionomycin activated T-cells by a transwell insert did not display enhanced HLA class II expression **(iii)**, indicating dependence on intercellular contact. All images are representative of at least 15 images for each condition.



Supplementary Figure 2: Activated bystander T-cells induce a shift in apparent molecular weight of CD63 in moDC

MoDC were incubated in the absence or presence of activated or non-activated fixed bystander T-cells as indicated. Cells were then lysed with Triton X-100 and proteins deglycosylated with either EndoH (NEB) or PNGase F (NEB) as indicated. CD63 was detected by Western blotting. The upper line and arrowhead indicate the migration position of CD63 in the lysate of moDC incubated with non-activated bystander T-cells. The middle line and arrowhead indicate the migration position of CD63 after incubation with activated bystander T-cells. Upon removal of high mannose glycans with EndoH, CD63 migrated slightly further into the gel to approximately the same position at both conditions, suggesting that complex glycosylation of CD63 was compromised in moDC that were incubated with activated T cells. Although only part of CD63 was sensitive to PNGase F, the identical mobility of deglycosylated CD63 (bottom line and arrowhead) supports the idea that CD63 is indeed differently glycosylated in response to moDC contact with activated bystander T cells. Of note, CD63 was not detected in T cells.





4

Dendritic cells release exosomes together with phagocytosed pathogens; potential implications for the role of exosomes in antigen presentation

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Submitted

Abstract

Dendritic cells (DCs) have the unique capacity to activate naïve T cells by presenting T cell receptor specific peptides from exogenously acquired antigens bound to Major Histocompatibility Complex (MHC) molecules. MHC molecules are displayed on the DC plasma membrane as well as on extracellular vesicles (EVs) that are released by DCs, and both have antigen-presenting capacities. However, the physiological function of antigen presenting EVs is still unclear. We here demonstrate that the release of small EVs by activated DCs is strongly stimulated by phagocytic events. We show that, concomitant with the enhanced release of EVs, a significant proportion of phagocytosed bacteria was expulsed back into the medium. High-resolution fluorescence microscopic images revealed that phagosomes were surrounded by EV marker-proteins. This suggests that exosomes may be formed by the inward budding into phagosomes, whereupon they are secreted together with the phagosomal content. These findings may have important implications for selective loading of peptides derived from phagocytosed pathogens onto exosome associated MHC molecules, and have important implications for vaccine design.

Introduction

Dendritic cells (DCs) are professional antigen presenting cells that use their innate immune functions to drive adaptive immune responses[1]. Once activated, for example upon recognition of components of invading pathogens by Toll-like receptors (TLRs), DCs have the unique ability to excise peptides from proteins of exogenous origin that are acquired by endocytic processes, load these peptides onto major histocompatibility complex (MHC) molecules, and present the resulting complexes to activate cognate naïve T cells. Like most cell types, DCs release heterogeneous populations of extracellular vesicles (EVs). EVs include microvesicles that are pinched off from the plasma membrane, and exosomes that are secreted by multivesicular endosomes[2–4]. DC-derived EVs carry MHC-peptide complexes that can activate T cells by their own right or after being recruited and in association with bystander DCs[5–7]. DC-derived EVs are heterogeneous in size, molecular composition, and capacity to stimulate T cells[8–10]. Their functionality is also dependent on the status of maturation of the producing DC[10–12]. EVs that were isolated from cultured DCs have been tested for their potential use as vaccine against cancer and pathogens[2,6,13–15]. Nevertheless, the precise role that DC-derived EVs play *in vivo* remains unclear. Previously, we reported that interactions of DCs with activated T-cells stimulated the release of antigen presenting EVs, suggesting a role EVs in dissemination and reinforcement of antigen presenting potential within lymphoid tissues[16,17]. Another hint for antigen presenting functions of EVs came from observations that both macrophages and epithelial cells can release phagocytosed pathogens through non-lytic expulsion, involving direct fusion of the phagosome with the plasma membrane[18–24]. Interestingly, it has been described that in the process exosomes may be co- released[25]. In our current study we demonstrate for the first time that in response to being challenged by *E.coli*, DCs are also capable of expulsion a significant proportion of phagocytosed *E.coli*. Moreover, we show that the expulsion coincides with the release of exosomes. Potentially, these observations have implications for preferential loading of pathogen-derived antigens onto exosome associated MHC molecules, and the efficacy of such generated exosomes in antigen presentation.

Experimental procedures

Cell culture and processing

Blood from healthy volunteers was obtained following institutional ethical approval (www.umcutrecht.nl/METC), METC protocol number 07-125/C. The experiments abide by the Declaration of Helsinki principles for human research ethics. Peripheral blood mononuclear cells (PBMCs) were isolated from lithium heparinized blood samples using

Ficoll isopaque density gradient centrifugation (GE Healthcare). CD14+ monocytes were isolated by positive selection using CD14+ MicroBeads (Miltenyi Biotec). The purity of CD14+ sorted cells was determined using flow cytometry after staining for CD14 and CD3. Only cultures containing ≥90% CD14+ cells prior to differentiation towards moDCs were used for further experimentation. For differentiation into moDC[26], CD14+ cells were cultured in 6-wells plates (Nunclon, Thermo Scientific) at a concentration of 1 to 1,5 x 10^6/ml in 2 to 3 ml per well at 37°C and 5% CO_2 in RPMI 1640 GlutaMAX (Gibco), 1% Penicillin/Streptomycin (Gibco) and 20% heat inactivated, 0,2 µM filtered FCS (Biowest), supplemented with 450 U/ml rhGM-CSF (Immunotools) and 300 U/ml IL-4 (Immunotools) for a total of five days. Cytokines were replenished after three days. Prior to experiments, the immature moDCs, which were in suspension, were harvested by subtle resuspension in culture medium, and washed once with PBS by centrifugation for 10 min at 330 x g. After washing, the immature moDC were resuspended in culture medium containing EV depleted FCS for further culture/experimentation. When indicated, moDC were cultured for 16 hours in the presence or absence of 100ng/ml ultrapure LPS (from *E. coli* strain O111:B4, Invivogen) and/or heat inactivated *E.coli* (see below). For these conditions, cells were seeded in 6 wells plates in 2ml/well at a density of 100,000 cells/ml. EV-depleted FCS was prepared by centrifugation after 5-fold dilution in RPMI for 18 hours at 100,000 x g in polyallomer tubes (Beckman Coulter) using a swing-out rotor (SW-28, Beckman Coulter).

E.coli preparation

E.coli strain DH5α was cultured in LB medium (MP Biomedicals) to a OD600 of 0.5 and then heat-killed by incubating for 30 min at 75°C. Heat killed bacteria were pelleted by centrifugation for 5 min at 13,300 x g, resuspended in 0,2M bicarbonate buffer at pH 8,3 using a 23G syringe, and washed in the same buffer using the same procedure. Washed bacteria were labeled either with Cy3B-NHS (200µg/ml, Thermo Fisher), or with a combination of AlexaFluor405-NHS (200µg/ml Thermo Fisher) and EZ-Link NHS-LC-Biotin (88µg/ml (equimolar), Thermo Fisher) for 45 minutes at 4°C under constant rotation. In case of double labeling, EZ-Link NHS-LC-Biotin was added 10 min after NHS-AlexaFluor405-NHS ester. After labeling, bacteria were washed three times by centrifugation for 5 minutes at 13,300 x g and resuspension in PBS (phosphate buffered saline, Gibco) containing 100mM glycine (Sigma). Glycine was added to ensure quenching non-reacted NHS-groups. Finally, the bacteria were washed three times in PBS prior to storage at 4°C. Bacteria were quantified using a hemocytometer.

Flow cytometry

After incubation, moDCs in suspension were harvested in excess ice cold PBS. Adherent cells were and gently dissociated by pipetting in ice cold PBS and pooled with the previous fraction. Viability was probed using 7-Aminoactinomycin D (7-AAD) (1:25, BD) according to manufacturer instructions. All subsequent immune-labelling steps were performed on unfixed cells at 4°C. Prior to incubation with antibodies, cells were blocked in 0.5% NMS (Fitzgerald 88R-M002) diluted in flow cytometry buffer (PBS with 0.5% BSA and 0.02% NaN3). HLA-II was detected with PE-Cy7-labeled mouse-anti human HLA-DR (clone L243; 1:200; BioLegend), CD86 with PE-labeled mouse anti-human CD86 (clone 2331/FUN-1, 1:50, BD), CD11c with fluorescein-5-isothiaocyanate (FITC)-labeled mouse anti-human CD11c (clone BU15, 1:50, Invitrogen), CD14 with FITC-labeled mouse anti-human CD14 (clone Tük 4, 1:100, Miltenyi), and CD3 with pacific blue (PB)-labeled mouse anti-human CD3 (clone UCHT-1, 1:50, Beckman Coulter). PE-labeled murine IgG1,k (clone MOPC-21; BD) and PE-Cy7-labeled murine IgG2a,k (clone MOPC-173; BioLegend) were used as isotype controls. Flow cytometry was performed on a FacsCanto II (BD) flow cytometer and the data were analyzed with FlowJo 10.0 software (Treestar).

Flow cytometry-based expulsion analysis

MoDCs were incubated for 3 hours with *E.coli* that were both biotinylated-, and labeled with AF405 (25 *E. coli* per moDC). The moDCs were then dissociated and resuspended in ice cold PBS, and collected by centrifugation at 240 x g for 4 minutes at 4°C in a tabletop centrifuge. At this centrifugation force the majority of non-phagocytosed *E.coli* remained in the supernatant. The pelleted moDCs were resuspended and washed in PBS by centrifugation at 240 x g, and probed for 10 minutes with Streptavidin-labeled allophycocyanin (APC) (1:50, eBiosciences) on ice to label any remaining extracellular non-phagocytosed biotinylated-, AF405-labeled *E.coli*. After washing away excess streptavidin-APC, the moDCs were re-incubated at cell culture conditions for another 3 hours to allow expulsion of phagocytosed APC-negative *E.coli*. Hereafter the samples were centrifuged for 4 min at 240 x g rpm to remove moDCs. Expulsed *E.coli* was collected from the supernatant by centrifugation at 13,300 x g for 8 min and labeled with streptavidin- phycoerythrin (PE) (1:50, eBiosciences) for 10 minutes on ice. After labelling, *E.coli* was washed in cold medium and PBS, and resuspended in flow cytometry buffer (PBS with 0.5% BSA and 0.02% NaN_3) for flow cytometric analysis, as described above.

EV isolation and Western blotting analysis

EVs were collected from culture media by differential (ultra)centrifugation at 4°C, as reported earlier[27]. Briefly, cells were removed in two subsequent centrifugation steps of

10 min at 200 x g. Next, supernatants were sequentially centrifuged two times for 10 min at 500 x g, once for 30 min at 10,000 x g, and finally for 65 min at 100,000 x g. The last two centrifugation steps were performed in polyallomer tubes (Beckman Coulter) using a swing-out rotor (SW-40, Beckman Coulter). The 10,000 x g and 100,000 x g pellets, predominantly containing large and small EVs respectively, were lysed in non-reducing SDS-PAGE sample buffer and incubated for 5 min at 100°C. After separation by 10% SDS-PAGE, proteins were transferred to 0.45 µm polyvinylidene difluoride membranes (Merck Millipore). The blots were blocked in PBS containing 0.2% cold water fish skin gelatin (Sigma) and 0.1% Tween-20. Immuno-labeling was performed in the same buffer using mouse anti-human CD9 (clone HI9a; 1:2000; Biolegend), mouse anti-human CD63 (clone TS63; 1:2000; Abcam), mouse anti-human CD81 (clone B-11; 1:400; Santa Cruz), mouse anti-human HLA-B,C (clone HC-10; 1:400; kindly provided by E.J.H.J. Wiertz), or mouse anti-human HLA class II (clone CR3/43; 1:10,000; Dako). Primary antibodies were labelled with HRP-conjugated goat anti-mouse IgG and IgM (1:10,000; Jackson). HRP activity was monitored using ECL (SuperSignal West Dura Extended Duration Substrate, Thermo Scientific) and detected with a ChemiDoc MP Imaging System (BioRad). Relative signal strengths were determined using Image Lab V5.1 (BioRad).

Structured Illumination Microscopy (SIM)

MoDC were cultured for approximately 16 hours either in presence or absence of Cy3B-labeled *E.coli* (10 *E.coli*/moDC) in 6-wells culture plates, and subsequently harvested in ice cold PBS. The harvested moDCs were pelleted by centrifugation for 10 minutes at 330 x g, and resuspended to 0.5 x 10^6 cell/ml in tissue culture medium. Next, 200 µl samples of the cell suspension were placed onto 12mm glass coverslips (World Precision Instruments) into a custom made adaptor (3D design available upon request) and fitted into cuvettes (Shandon-Elliott) that were pre-wetted with 50 µL 2% bovine serum albumin in PBS, and centrifuged for 3 min at 800 rpm and room temperature using a cytocentrifuge (Shandon-Elliott). Subsequently, the coverslips were fixed for 30 min in 4% paraformaldehyde in 0,1M phosphate buffer, pH 7,4. Fixed cells were washed with PBS and permeabilized for 5 minutes in PBS containing 0,1% Saponin (Sigma), 2% bovine serum albumin (BSA), and quenched for 20 minutes in 20mM NH_4Cl. Subsequent washing and labeling was performed in PBS containing 2% BSA and 0.1% saponin. Cells were labeled with mouse anti human HLA class II (CR3/43; 0.4 µg/ml, Dako) or mouse anti human CD63 (H5C6, 1µg/ml, BD) for 45 min at room temperature, washed three times and incubated with goat anti-mouse AlexaFluor488 (1 µg/ml, Invitrogen). Labelled cells were washed twice in labelling buffer, twice with 0.22 µm filtered PBS, followed by a washing step in deionized water. Coverslips were mounted onto object glasses in Prolong Diamond embedding media (Thermo Fisher) and left to solidify overnight.

Images were acquired using a DeltaVision OMX V4 Blaze imaging system in SIM imaging modus with a 60x objective (Olympus U-PLAN APO, NA 1.42) and oil with a refractive index of 1.516, with the factory-supplied BGR filter tray and 125 nm step size in the Z-axis. Acquisition was executed by the OMX Deltavision software package and images were reconstructed using SoftWoRx (GE Healthcare) with OTFs generated by GE Healthcare service engineers (raw data, alignment parameters available on request). Figures were prepared in Imaris 8.1 (Andor/Bitplane) with linear intensity adjustments for labelled channels and a gamma correction of 1.4 was applied for *E.coli* channels to highlight intracellular fragmented bacteria.

Live confocal microscopy-based expulsion analysis

MoDCs were labeled with cell trace yellow (CTY) (1µM, Thermo Fisher) in PBS at room temperature for 5 minutes, followed by addition of cold culture medium 1:1 and a further 5 minutes incubation on ice. Subsequently, cells were pelleted by spinning 10 minutes at 330 x g and resuspended in warm medium. Next, the moDC were seeded in Eppendorf tubes and incubated with biotinylated, AlexaFluor405-labeled *E.coli* (10/moDC) at regular culture conditions. After three hours, moDCs were pelleted by centrifugation at 240 x g for 4 minutes and washed two times with cold PBS, followed by staining with AlexaFluor647-labeled streptavidin (1:100, Invitrogen) for 10 minutes on ice. Subsequently, cells were washed twice with cold medium, resuspended in cold medium and seeded in a Fluorodish (35-100, World Precision Instruments). The Fluorodish was kept on ice until the start of imaging. Imaging was performed on a NIKON A1R confocal microscope with a 40x Plan Apo objective (NA 1.3) while maintaining cell culture conditions (37°C, 5% CO_2) in a tabletop culture control unit (TOKAI Hit). Confocal scanning was performed using the resonance mode with a pinhole set to 130.27 27µm and a pixel size of 0.62 µm and 4x airy disk. Diode laser and filter settings were used to detect AlexaFluor405, CTY, and AlexaFluor647 sequentially using bidirectional line switching. Differential interference contrast (DIC) images were collected along with the CTY channel. Overviews of the cultures were generated every 2 minutes by automated scanning of 4x4 image fields in 5 positions along the Z-axis at 1.5 µm steps. Imaging data were acquired over a period of up to 11 hours and processed in NIS elements 5.02 (Nikon Microsystems). Images were pre-processed by subtracting the average intensity in the Z-stack before maximal internal projection. Objects were isolated using the spot isolation procedure in NIS elements, using background subtraction and median filtering. Alexa647 binary isolates were dilated 1 pixel to compensate spectral and motion shifts due to the sequential recording. Isolated objects were classified based on co-incidence of the channels. Expulsed *E.coli* objects were defined as Alexa405-positive, Alexa647-negative, CTY-negative events, counted over time, normalized to number of counted

moDCs, and related relative to the average total Alexa 405-positive, Alexa647-negative events (both CTY-positive and CTY-negative) measured in the first hour of imaging.

Statistical analysis
Significance of difference was determined using Wilcoxon's signed rank test with GraphPadPrism 7 software. P values ≤0.05 were considered statistically significant.

Results

MoDCs phagocytose *E.coli* and become activated in the process
To study long-term effects of phagocytosis on the release of EVs by moDCs, we used fluorescently (AF405) labeled *E.coli* that were heat killed to prevent overgrowth of moDCs. CD11c-expressing moDCs were detected by flow cytometry **(figure 1A)**. The viability of the moDCs, as determined by 7-AAD-exclusion, was unaffected after 24 hours incubation in the presence of 25 bacteria per moDC (**figure 1B**). HLA-DR and CD86 were increasingly expressed over time, and nearly all moDCs had matured after 24 hours (**figure 1C-E**). Approximately 50% of the moDCs contained a measurable signal for phagocytosed *E.coli* after 8 hours incubation with 25 bacteria per moDC, and this did not further increase over time (**figure 1C**). Of note, this percentage was significantly lower when moDCs were incubated with fewer *E.coli* (**figure 1F**). The amount of 25 E.coli/ moDC provided a maximal activation signal, as determined by the observation that the additional presence of LPS did not further enhance surface expression of moDC maturation markers (**Supplementary figure 1**).

Phagocytosis of *E.coli* by the moDCs was confirmed using super-resolution fluorescence microscopy 3D imaging (SIM), wherein moDCs were stained for HLA class II or CD63 (**figure 2)**. In immature, unstimulated moDC (left panel figure 2A), HLA class II (green) was mostly intracellular. As expected, incubation with *E.coli* (purple), resulted in transfer of HLA class II to the plasma membrane. Importantly, phagocytosed *E.coli* was often found surrounded by a ring of small HLA-II labelled puncta (arrows in right panel) (**figure 2A**, **and supplementary movie 1** for 3D rotation). Similar to HLA class II, CD63 (green) was found throughout the cytosol in immature moDCs. However, upon phagocytosis of *E.coli*, CD63 redistributed to structures surrounding the *E.coli* containing phagosomes. (**figure 2B, and supplementary movie 2** for 3D rotation). CD63 is known to be highly enriched in endosomes as well as in exosomes[28] and thought to be instrumental for cargo selection into exosomes[28,29], triggering the question whether exosomes may be formed as intraluminal vesicles in phagosomes.

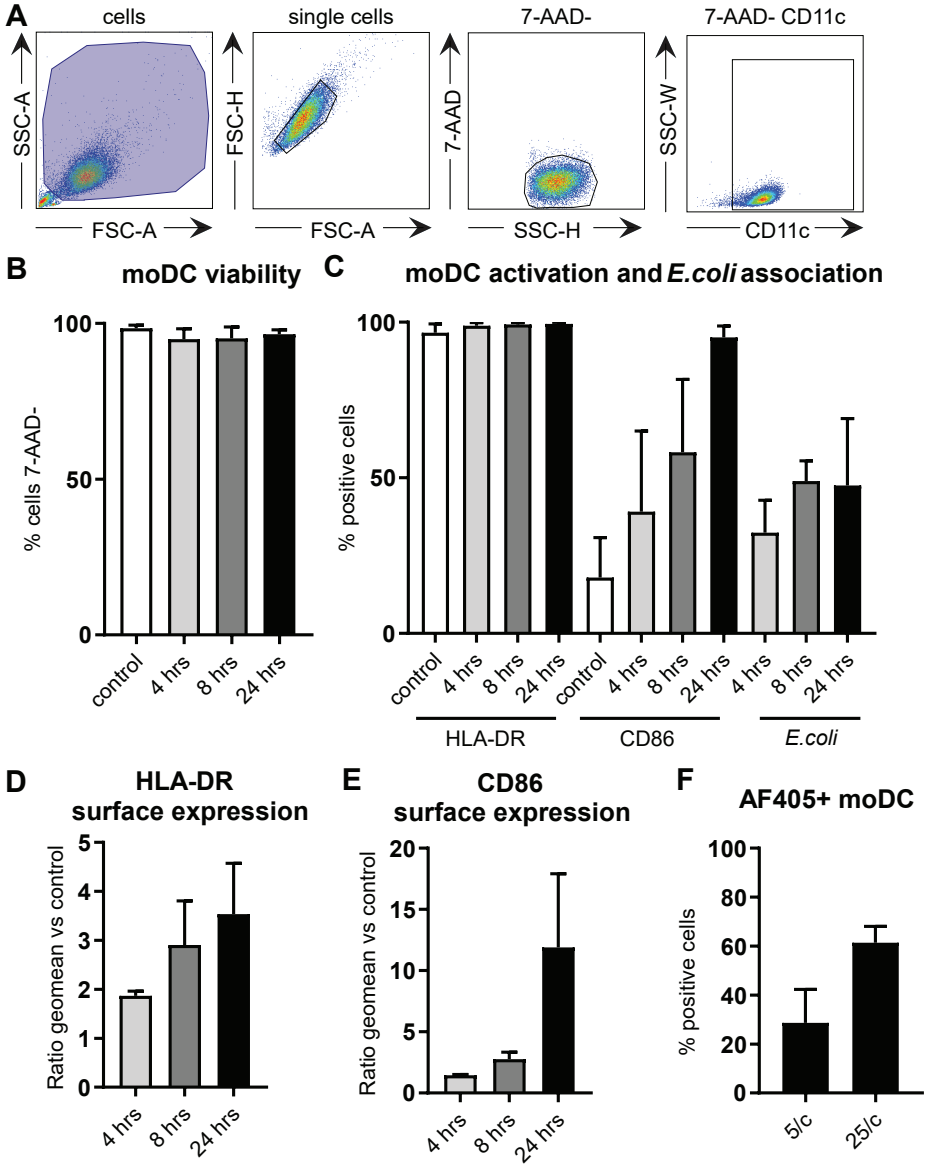


Figure 1: Phagocytosis of E.coli and activation of moDCs
(**A**) Flow cytometric gating strategy for moDCs. Cells were first selected on forward and side scatter properties, followed by selection of single cells (FSC-H vs FSC-A). Subsequently, viable single cells were identified by exclusion of 7-AAD. Finally, moDCs were selected based on cell surface expression of CD11c. (**B-E**) moDCs were incubated for 24 hours in the absence (control) or presence (4, 8, or 24 hours) of AF405 labelled *E.coli* (10 bacteria per moDC), harvested, stained with antibodies, and analyzed by flow cytometry as in A. n = 3, mean ± S.D. (**B**) Viability of moDCs was unaffected by incubation with *E.coli*.

Figure 1: Continued

(**C**) All moDCs expressed HLA-DR on their surface, irrespective of activation by *E.coli*. The percentage of activated moDCs, as determined by expression of CD86, steadily increased up to 95% up to 24 hours of incubation with *E.coli*. The percentage of moDCs associated with *E.coli* increased up to 50% at 8 hours incubation, and did not increase further. (**D** and **E**) Geometric mean of HLA-DR and CD86 cell surface expression after incubation with *E.coli* relative to control. (**F**) Percentage of *E.coli* labeled moDCs after 24 incubation with either 5 or 25 *E.coli* per moDC. n = 3, mean ± S.D.

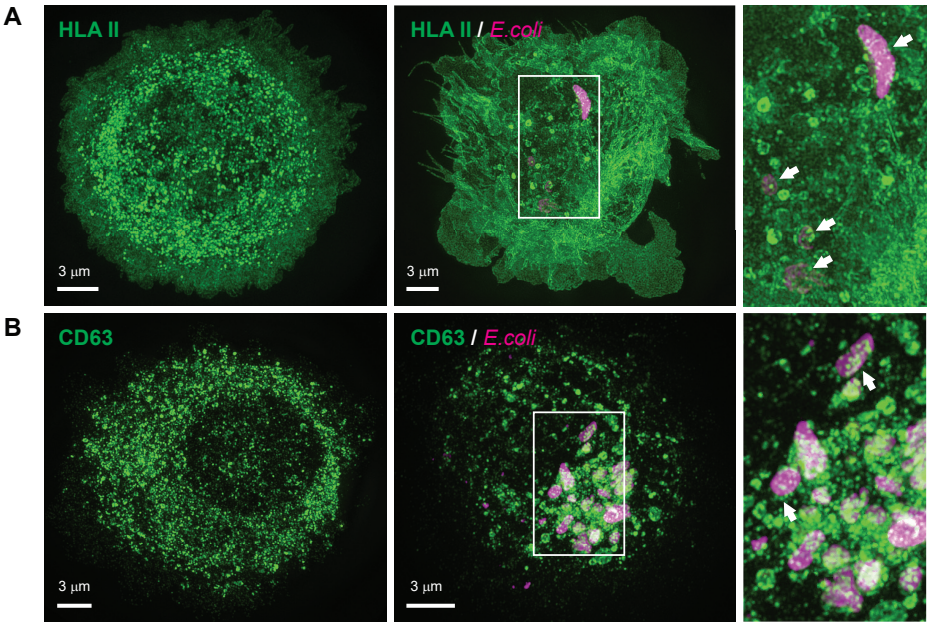


Figure 2. Subcellular distributions of HLA class II and CD63 relative to phagocytosed E.coli. The pictures are representative flattened 3D SIM images from one experiment out of three independent experiments. **A** The subcellular distributions of HLA class II (green) in an immature moDC (left panel) and in an activated moDC after uptake of *E.coli* (purple) (middle panel). Note the redistribution of HLA class II from endosomal/lysosomal compartments to the plasma membrane in response to activation. The indicated segment is enlarged in the right panel. Note the punctate HLA II staining surrounding *E.coli*-containing phagosomes (4 bacteria indicated with arrows). (**B**) The subcellular distributions of the exosomal marker CD63 (green) in an immature moDC (left panel) and in an activated moDC after uptake of E.coli (purple) (middle panel). The indicated segment is enlarged in the right panel. Note the recruitment of CD63 towards the phagosome containing area of the moDC, and the punctate staining pattern surrounding the many *E.coli*-containing phagosomes.

Expulsion of phagocytosed *E.coli* by moDCs

When exosomes can indeed be formed as ILV in phagosomes, their release by secreting phagosomes would imply that phagocytosed *E.coli* can be secreted back into the extracellular environment. To investigate this possibility, we first used a flow cytometry-based approach (schematic representation in **figure 3A**). First, moDCs were incubated for 3 hours with *E.coli* that were both AF405-labelled and biotinylated. The moDCs were then separated from the majority of non-phagocytosed extracellular *E.coli* by centrifugation. Those bacteria that were not phagocytosed but co-pelleted with the moDCs (either in association with the moDC plasma membrane or as a free bacterium) were labelled with streptavidin-APC, leaving truly phagocytosed *E.coli* unlabeled. The moDCs were then chased for 3 hours at 37°C allowing expulsion of phagocytosed, APC-negative, *E.coli*. After cooling, expulsed *E.coli* were separated from their originating moDCs and collected by differential centrifugation, stained with streptavidin-PE, and identified by flow cytometry as $AF405^+$, APC^-, PE^+ events. The $AF405^+$ gating strategy is illustrated for a control *E.coli* sample that was labelled in the absence of moDCs, demonstrating the efficacy of labeling (**figure 3B and 3C, left panel**). With this strategy, we detected a small but significant population of *E.coli* that had been expulsed after being phagocytosed by moDCs (**figure 3C, right panel**). This value reflects the amount of recycled *E.coli* relative to the amount of *E.coli* that was not phagocytosed and failed to be removed after pulse loading the moDCs. Therefore, although this approach demonstrated that expulsion exists, it did not enable us to quantify the relative amount of phagocytosed *E.coli* that was expulsed.

For quantification we resorted to live cell fluorescence microscopy. Cell Trace Yellow (CTY)-labelled moDCs were pulsed for 3 hours with biotinylated AF405-labelled *E.coli*. Subsequently, the moDCs were washed, after which remaining extracellular *E.coli* were labeled at 4°C with streptavidin-AF647. The moDCs were then chased up to 11 hours with continuous imaging (**figure 4A**). Automated logical gating with different color-combinations was used to identify and follow individual events during the chase (**figure 4B**). Phagocytosed *E.coli* were defined inside moDCs as individual events in a CTY-positive background that were both AF405-positive and AF647-negative. AF405-positive *E.coli* that were also labeled with AF647 were considered not to have been phagocytosed directly after pulse loading. Phagocytosed AF405-positive, AF647-negative *E.coli* was identified to be expulsed" when appearing outside CTY-labelled moDCs during the 11-hour chase. The percentage of phagocytosed *E.coli* that was expulsed increased over time, reaching 32.2±15.1% (mean ± SEM from 3 independent experiments) after 11 hours (**figure 4C**).

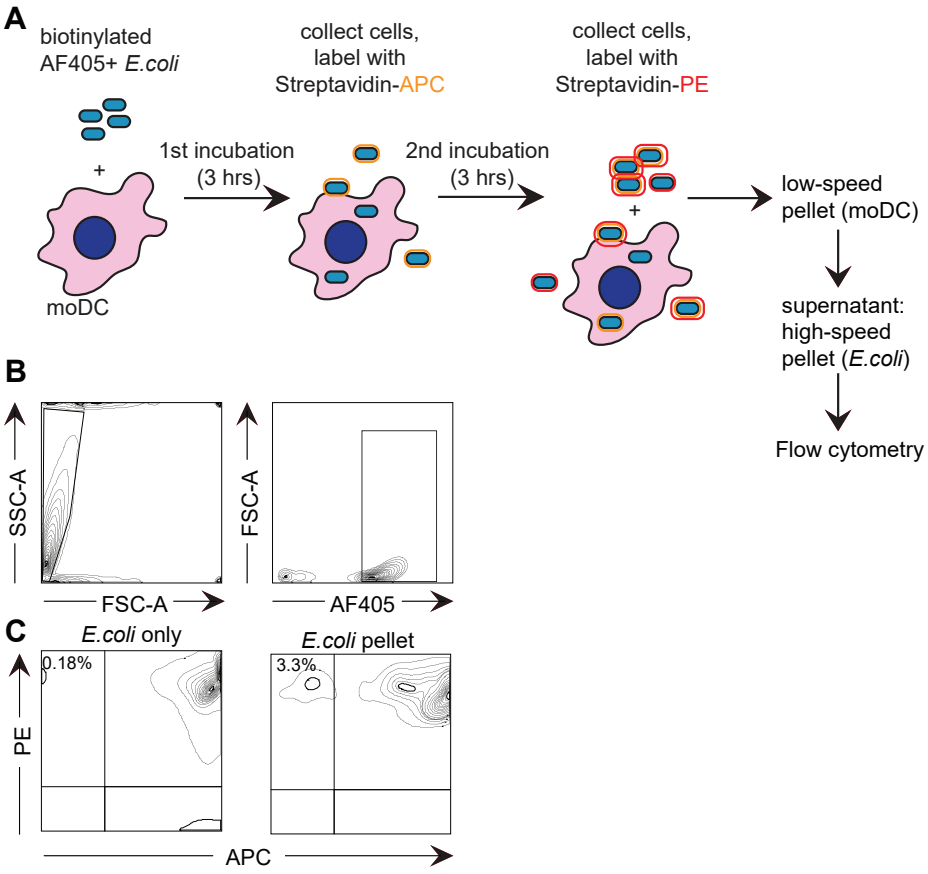


Figure 3: Flow cytometry-based analysis of E.coli expulsion by moDCs
(**A**) Schematic presentation of the experimental design. MoDCs were incubated for 3 hours with *E.coli* that was labeled with both biotin and AF405 (blue). Subsequently, all moDCs and some *E.coli* were collected by centrifugation for 8 minutes at 260 x g and stained with streptavidin-APC (orange), labelling those biotinylated *E.coli* that were co-pelleted with the moDCs but not phagocytosed. Subsequently, moDCs were incubated for another 3 hours at 37°C, cooled to 4 °C, and stained with streptavidin-PE (red). The moDCs together with some *E.coli* were removed by centrifugation at 260 x g, and *E.coli* remaining in the moDC supernatant was pelleted by a subsequent centrifugation step at 13,300 x g. Expulsed *E.coli* was identified by flow cytometry as $AF405^+$, APC^-, PE^+ events. (**B**) Gating strategy for *E.coli: E.coli*-sized particles were gated based on scatter and AF405 signal. (**C**) Flow cytometry plots showing labeling efficiency in a control ("*E.coli* only", left panel) and expulsed *E.coli* (APC^-, PE^+, "*E.coli* pellet", right panel). Representative for a total of 3 independent experiments.

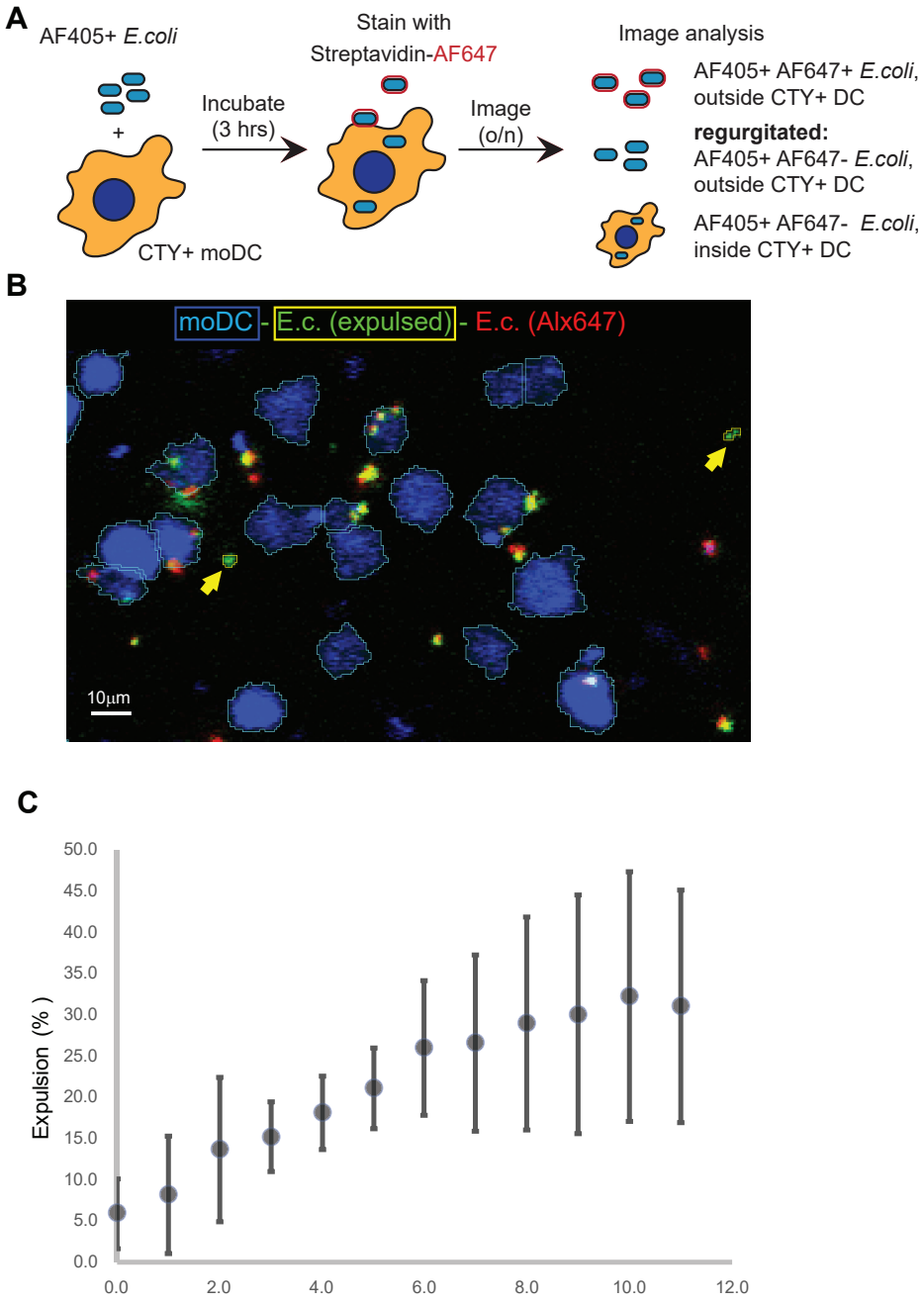


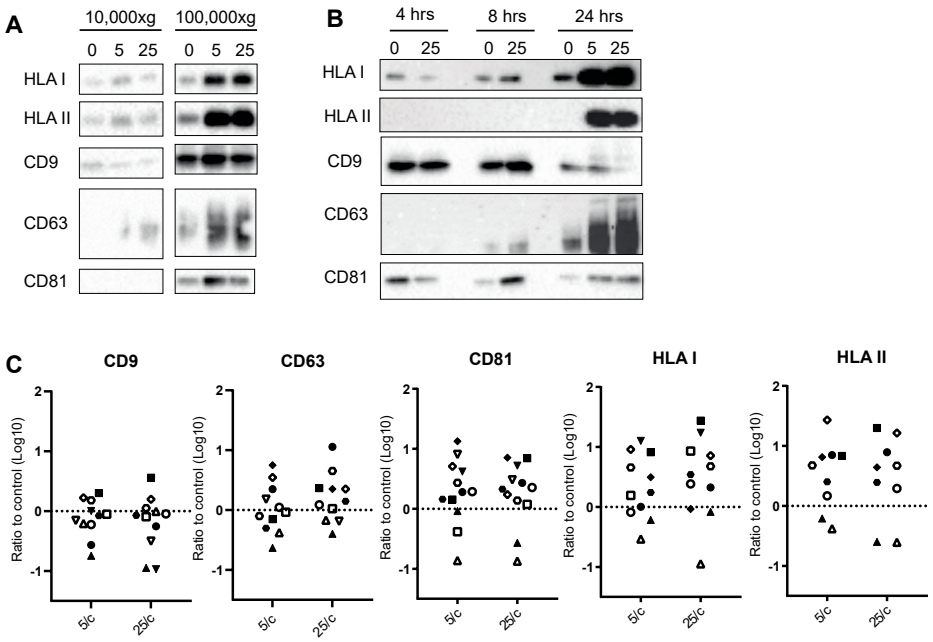
Figure 4: Confocal microscopy-based dynamic analysis of E.coli expulsion
(**A**) Schematic presentation of the experiment. CTY-labeled moDCs were incubated for 3 hours with *E.coli* that were labelled with both biotin and AF405. Subsequently, moDCs were pelleted, labelled on ice with streptavidin-AF647, and washed.

Figure 4: Continued

The cells were transferred to a confocal microscope. The cells were followed up to 11 hours in a 5% CO_2 containing atmosphere at 37°C using automated confocal imaging. CTY, AF405 and AF647 and combinations thereof were detected at a 2-minute intervals and analyzed. (**B**) Representative still image at 10 hours from one out of three independent experiments. MoDCs are depicted in blue, cell boundaries were automatically drawn. Phagocytosed *E.coli* is green in a blue background, expulsed *E.coli* is represented by green areas that are encircled outside blue areas and indicated by the yellow arrows. *E.coli* that was not phagocytosed after pulse loading the moDCs is stained both red and green. *E.coli* that was not phagocytosed after pulse loading but during the chase is represented by the red dots in a blue background. (**C**) Kinetics of *E.coli* expulsion. *E.coli* counts were normalized to moDC counts. Expulsed *E.coli* ($AF405^+$, $AF647^-$ in a CTY^- background) was determined as a percentage relative to all $AF405^+$, $AF647^-$ *E.coli* (within and outside CTY background). The plot represents data from three independent experiments (mean ± SEM).

Phagocytosis induced release of EVs

We next set out to test whether phagocytosis of *E.coli* induced the release of EVs by activated moDCs. To this end, moDCs were activated by LPS, either in the absence or presence of 5 or 25 heat inactivated *E.coli* per moDC. Activation of moDCs by LPS alone may already affect the release of EVs, although this idea is still controversial since only minor stimulatory as well as minor inhibitory effects of LPS on EV release by DCs have been reported[30,31]. Therefore, to eliminate any potential bias introduced by moDC activation on EV release, LPS only was taken as control condition. EVs were collected from the culture media by differential centrifugation. After removal of cells and cell debris, large EVs were pelleted first by centrifugation at 10,000 x g. The supernatant was then re-centrifuged at 100,000 x g to collect small EVs, including exosomes[12,27]. The 10,000 x g and 100,000 x g pellets were analyzed by immunoblotting for the presence of EV-associated proteins. The antigen presenting molecules HLA-I and HLA-II, and the tetraspanins CD9, CD63, and CD81, were more abundantly present in the 100,000 x g compared to 10,000 x g pellets (**Figure 5A**). This observation is consistent with the predominant association of these markers with relatively small EVs[12]. Interestingly, the presence of LPS + E.coli stimulated the release of these markers, as compared LPS alone, indicating stimulation by the phagocytosed bacteria independently of moDC activation. To determine the kinetics of secretion, moDCs were stimulated for 4, 8 or 24 hour with LPS only or in the additional presence of 5 or 25 *E.coli* per moDC. HLA class-I and -II and CD63 were most abundantly present in the 100,000 x g pellet after 24 hours incubation with *E.coli*, whereas CD9 did not increase over time (**Figure 5B-D**).



D

Marker	5/c	25/c
CD9	0.94 (±0.58)	0,997 (±0.98)
CD63	1.91 (±1.93)	3.08* (±3.52)
CD81	3.47* (±3.86)	2.85* (±2.38)
HLA class I	3.96* (±4.18)	6.83* (±8.43)
HLA class II	6.34* (±8.19)	6.46* (±7.09)

Figure 5: *E.coli* -induced EV release by moDCs
(**A**) Representative immunoblot from 11 independent experiments detecting HLA-I, HLA-II, CD9, CD63, and CD81 in EVs collected by sequential 10,000 x g and 100,000 x g centrifugation from the culture media from moDC that were incubated for 24 hours in the presence of LPS only (0) or presence of both LPS and 5 or 25 *E.coli* per moDC. Samples of the 10,000 x g and 100,000 x g pellets were loaded and detected on the same blot (exemplified in **supplementary figure 2**). (**B**) Representative immunoblot of EV markers in 100,000 x g pellets from culture media from moDCs that were incubated for 4, 8, or 24 hours either in the presence of LPS only (0) or in the presence of both LPS and 5 or 25 *E.coli* per moDC. **(C)** Quantification of signals as in (B) from 11 independent experiments of appropriately exposed immunoblots of EVs pelleted at 100,000 x g from culture media of 24 hour treated moDCs. Each symbol corresponds to 1 individual donor. Signal strength induced by 5 or 25 *E.coli* per moDC is plotted relative to the signal in the absence of *E.coli* within the same experiment, and fold increase is expressed on a log10 scale. (**D**) Statistical analysis of signals in C (mean ± SD; * indicates difference with p≤0.05).

These data were collected from 11 independent experiments, in which cells from different donors were used. The high variability between these experiments may be due donor differences. Alternatively, it should be noted that, although only batches of moDC with >90% viability were selected for these experiments (using 7-AAD as in figure 1B), the release of EV from a minor population of dying cells can result in a dramatic increase in background signal for EV release. Indeed we observed that signals for EV markers increased, predominantly in 10,000 x g pellets, when moDC preparations with viability scores < 90% were used (data not shown). The majority of EVs released by lesser quality moDC may represent apoptotic vesicles, and the high background signals imposed by such apoptotic bodies masked contributions of EV that were released in response to *E.coli* phagocytosis. It is likely that even in our moDC preparations selected for high viability scores, slight variation in the quality of the cell cultures contributed to scatter between the 11 replicates. Nonetheless, we found a dose dependent (5 versus 25 *E.*coli per moDC) and significant increase for CD63, CD81, HLA class I, and HLA II in 100,000 x g pellets in response to *E.coli* + LPS, as compared to LPS alone (**figure 5D**). In contrast, the release of CD9 remained unaffected. This is consistent with the notion that CD9 may be predominantly associated with plasma membrane-derived microvesicles rather than with exosomes[12]. Importantly, the amount of EV associated HLA-I, HLA-II, CD81 and CD63, also increased with time (**figure 5B**), consistent with the rate of expulsion of phagocytosed *E.coli* (**figure 4C**), suggesting that exosomes may be expulsed by *E.coli* containing phagosomes that fuse with the plasma membrane.

Discussion

We here demonstrate that up to ~30% of *E.coli* that were phagocytosed by moDCs were expulsed back into the culture medium (**figure 4**), and that phagocytosis by these cells stimulated the release of EV associated markers up to six fold with comparable kinetics (**figure 5**). The concomitant release of phagocytosed *E.*coli and small EVs, together with the accumulation of HLA class II and CD63, a well-established exosome marker, in discrete puncta within the perimeter of E. coli containing phagosomes (**figure 2**), indicates that the released EVs were generated as ILV by inward budding into phagosomes, and secreted as exosomes as a consequence of fusion of phagosomes with the plasma membrane. The tetraspanin CD9 is thought to be primarily associated with MV rather than with exosomes[12]. In contrast to CD63 and CD81, the release of CD9 was not stimulated by E.coli (**figure 5**), consistent with the classification of the EVs as exosomes. We were unable to directly visualize secretion of exosomes from phagosomes, as the required technologies are not advanced enough yet. We attempted but failed to detect phagosome-plasma membrane fusion using tetraspanin-based pH-

sensitive optical reporters in combination with live total internal reflection fluorescence microscopy, a technique that was recently developed by Verweij and coworkers to demonstrate exosome secretion by MVB[32]. This technique relies on sensing a shift in pH of the environment by the pH sensitive fluorescent probe when expelled from acidic endocytic compartments into a pH neutral extracellular environment. However, acidification of phagosomes in DCs is limited by NOX2[33], and TLR4 engagement restrains phagosome fusion with lysosomes, to prevent full antigen degradation and promote cross-presentation[34]. This method thus cannot be applied to monitor the fusion of pH neutral phagosomes with the plasma membrane. Consistent with our observations for moDC, DCs protruding into the gastrointestinal epithelium have been shown to secrete apically phagocytosed E.coli at their basolateral side[35]. The concept of non-lytic extrusion of phagosomal content has also been demonstrated for macrophages after phagocytic uptake of Cryptococcus neoformans[21,24], Candida albicans[23], or yeast[22]. Similar to phagosomes, also autophagosomes can secrete their contents. For example, uropathogenic *E. coli* was demonstrated to be expelled from infected bladder epithelial cells through expulsion of the content of autophagosomes[36]. In the same study, transmission electron microscopic pictures revealed the presence of small vesicles within the autophagosomal lumen, between its delimiting membrane and the autophagocytosed pathogen, consistent with a pre-exosomal identity[36]. Similar to phagosomes in DCs, these autophagosomes have a neutral pH, which prevents degradation of their content by lysosomal proteases and stimulates exocytosis via TRP channel 3 (TRPML3), a transient receptor potential cation channel, resulting in expulsion of the bacteria together with associated exosomes[36]. Triggering of TLR4 in these autophagosomes stimulates polyubiquitination of TRAF, which on its turn stimulated RalGDS, a guanine nucleotide exchange factor (GEF), to assemble the exocyst complex[37]. Whether similar mechanisms drive the extrusion of phagosomes by DCs or other cell types remains to be established. However, the release of exosomes by gastroendothelial cells was stimulated by infection with the protozoan parasite Cryptosporidium parvum, in a process that involves TLR4/IKK2 signaling and a SNAP23-dependent exocytosis[18]. Interestingly, the secretion of exosomes by MVB also relies on the plasma membrane SNARE SNAP23[32], suggesting parallel mechanisms of exosome secretion by MVB and phagosomes.

DC-derived EVs are capable of inducing T-cell responses, through presentation of antigenic peptides on MHC molecules[2,4,5,10,11,17,38,39]. We recently demonstrated that non-cognate interactions between DC and bystander T-cells modulates third party antigen-specific T-cell responses via EVs, possibly also involving EV mediated transfer of miR-155[17]. The microRNA miR-155 is a critical regulator of adaptive immune responses[40],

exemplifying a mechanism of how antigen presentation via intercellular transfer of EVs may promote adaptive immunity.

Our current finding that exosomes can be generated in and secreted by phagosomes adds a new perspective of how exosomes may contribute in antigen presentation. MHC molecules in pathogen loaded phagosomes can be expected to be preferentially loaded with pathogen derived peptides, as compared to MHC molecules residing in the other hundreds or even thousands of compartments within the endocytic tract. As a consequence, also exosomes that are generated as ILV in phagosomes are preferentially loaded with pathogen derived peptides. In contrast, MHC molecules that are displayed at the plasma membrane are generically recruited from all intracellular compartments, and thus preferentially loaded with self-peptides. Hypothetically, this could be tested by mass spectrometry analysis of the peptidome presented by MHC molecules from isolated exosomes versus plasma membrane[41,42]. However, this is technically challenging, if not impossible at this time, given the limitations for obtaining sufficient quantities of exosomes from human moDCs as source for peptide-MHC class I and II complexes. Moreover, separation of exosomes from plasma membranes is limited by the fact that many EVs remain associated with the plasma membrane of their originating cell, possibly via tetherin[43], or as a result of recruitment by integrin binding[44]. Further optimization of the separation of exosomes and plasma membranes, as well as of the isolation of peptide-MHC complexes from exosomes, and the sensitivity of MS techniques may enable such experiments in the future.

In conclusion, we hypothesize that MHC molecules on exosomes that are secreted by phagosomes are preferentially loaded with pathogen-derived peptides, and thus excellently suited to stimulate adaptive immune responses. Importantly, T-cell receptors undergo dimerization before activation and that this property might be essential for T-cell activation[45]. Consistent with this idea, it has been proposed that dimerization of MHC class II molecules might be critical for TCR dimerization and T-cell activation[46,47]. However, given that T-cell activation can be efficient when less than 0.1% of the MHC class II molecules are loaded with a TCR-specific peptide[48], the probability that two identical peptide–MHC complexes are present at the same microdomain at the plasma membrane is neglectable. Preferential loading of peptide derived peptides onto exosome associated MHC class II may solve that problem, as this would both increase the density and force proximity of such pathogen peptide-MHC class II complexes. This would apply to DC exosomes that remain associated to the plasma membrane of their producing cell as well as for exosomes that are recruited by bystander antigen presenting cells, resulting in rapid dissemination of the immune response.

Funding
This work has been supported by project number 022.004.018a, which is financed by the Netherlands Organisation for Scientific Research (NWO), and a seed grant awarded by Utrecht University to WS and MB

Conflict of interest statement
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

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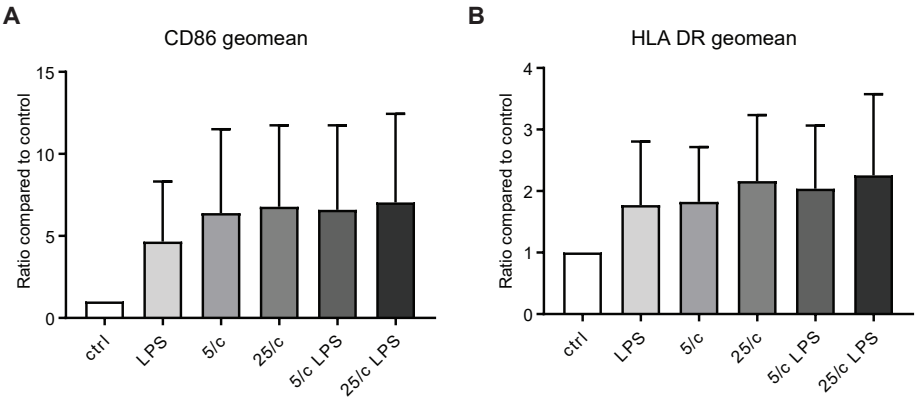
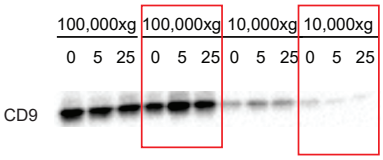
Supplementary figures

Supplementary figure 1: CD86 and HLA II surface expression *E.coli* +/- LPS



CD86 and HLA class II cell surface expression
MoDC were incubated with LPS or *E.coli* (5 or 25 per cell) alone, or a combination of LPS with *E.coli*. Cells were incubated with stimuli for 24 hours. The ratio of the geometric mean was calculated relative to the control condition. Combination of LPS with *E.coli* does not seem to increase the geomean compared to *E.coli* alone. Average of three independent experiments.

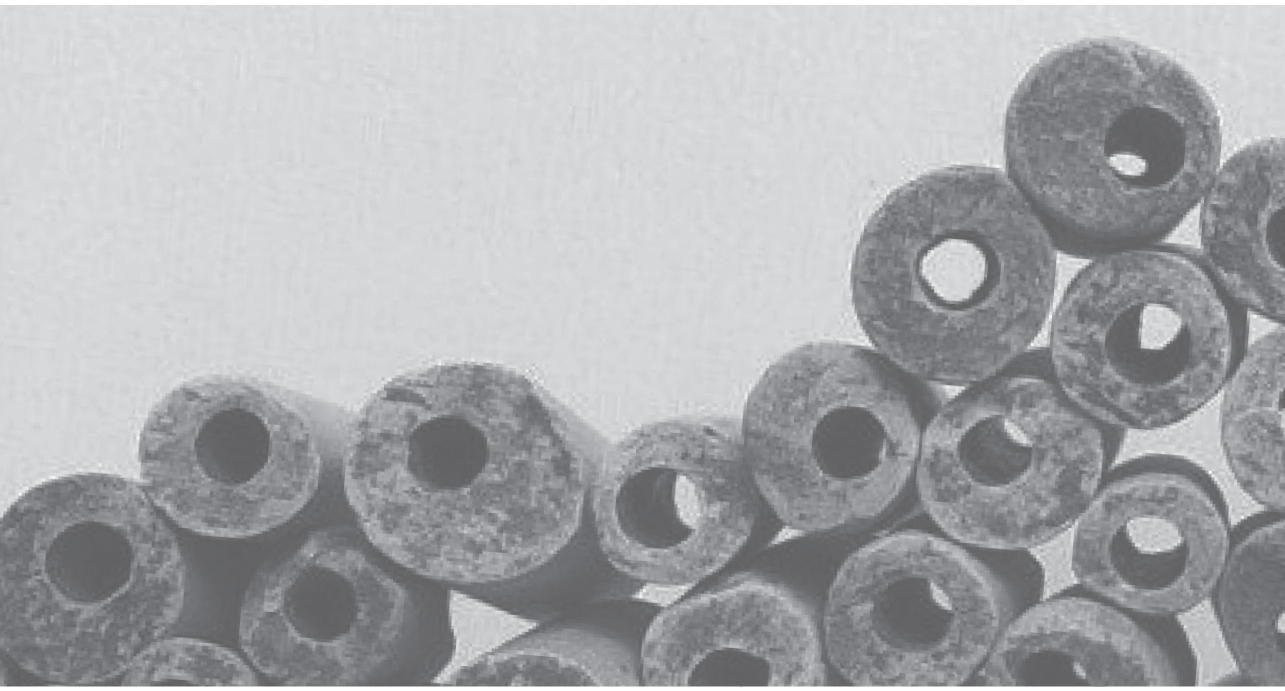
Supplementary figure 2:



Example of P4 versus P3 (CD9) figure 5A.
P3 and P4 samples are present on the same blot.

Supplementary movie 1: 3D rotation SIM image HLA II

Supplementary movie 2: 3D rotation SIM image CD63



5

Impaired proteolysis of CD74 by SPPL2a provokes a humoral response towards CD74 in ankylosing spondylitis

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Submitted

Abstract

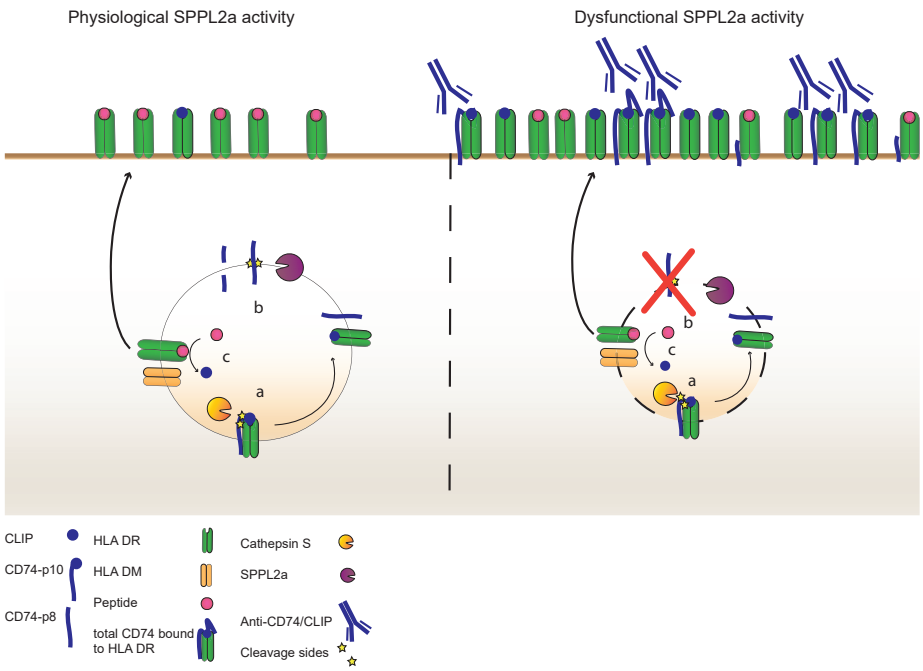
Objective: Ankylosing spondylitis (AS) is associated with autoantibody production to Class II MHC-associated invariant chain peptide, CD74/CLIP. Mechanisms contributing to this antigen-specific autoimmunity are unknown, but involves increased immunogenicity to the self-antigen CD74/CLIP. We examined the role of the CD74 processing enzyme signal peptide peptidase-like 2A (SPPL2a) in modifying the subcellular location of self-antigen CD74/CLIP, as a possible contributor to this antigen-specific autoimmunity.

Methods: We analyzed monocytes from healthy controls (n=47), psoriatic arthritis (n=27), rheumatoid arthritis (RA; n=16) and AS patients (n=18) for increased sensitivity to SPPL2a selective inhibitor $(Z\text{-}LL)_2$-ketone, as observed by CD74-p8 N-terminal fragment (NTF) accumulation. We measured endosomal and surface presence of full-length CD74, CLIP and HLA Class II using monocytes and SPPL2a-deficient THP1 cells, by flow cytometry and by confocal microscopy.

Results: We observed that monocytes from AS patients are increased sensitive to $(Z\text{-}LL)_2$-ketone compared to monocytes from healthy donors in terms of increased intracellularly accumulate CD74-p8 N-terminal fragments. AS monocytes show increased cell surface-display of HLA class II and full-length CD74, which we confirmed in SPPL2a-deficient THP-1 cells. Moreover, sera from AS patients contain IgG antibodies directed towards CD74 fragments present in SPPL2a KO THP-1 lysates.

Conclusion: We propose that the reduced activity of SPPL2a protease in monocytes of AS patients predisposes to CD74 endosomal accumulation. Accordingly, increased amounts of endosomal CD74/CLIP is deposited at the plasma membrane upon IFNγ exposure, yielding enhanced CD74/CLIP display and immunogenicity. As such, our findings explain how decreased activity of SPPL2a proteolysis can underlie anti-CD74 autoantibody formation in patients with AS.

Key words: *Ankylosing spondyloarthritis, SPPL2a, CD74, invariant chain, autoimmunity*



Graphical abstract

Left side, physiological situation: CD74 bound to MHC class II is cleaved by cathepsin S, leaving CLIP bound to MHC class II. CLIP is replaced by an antigenic peptide and the peptide-MHC class II complex is transported to the cell surface. SPPL2a is responsible for the degradation of CD7-p8. Right side, SPPL2a dysfunction: upon an inflammatory signal, CD74-p8 is not cleaved. As a consequence, late endosomes become compressed and facilitate the surface-directed transport of MHC class II, full-length CD74 and CD74-p8 remnants. An increase in display provokes the immune system to produce auto-antibodies to CD74.

1.1 Introduction

The spondyloarthropathies encompass a spectrum of rheumatic diseases subdivided in axial and peripheral spondyloarthritis (SpA) that include both ankylosing spondylitis (AS) and psoriatic arthritis (PsA) [1]. SpA is often considered an autoinflammatory condition – caused by repetitive trauma injuries and subsequent inflammatory responses – whereas the chronic inflammatory joint disease rheumatoid arthritis (RA) is considered an archetypical autoimmune disease [2, 3]. Whereas RA and SpA can share features such as destructive and chronic inflammation of the joints, the overall clinical presentation of these diseases is quite distinct. The latter suggest that these conditions share final common pathological pathways, but at the same time have unique molecular mechanisms causative to their specific phenotypes. At the molecular level, both SpA and RA are similar in showing linkage at genome-wide significance to chromosome 6p21, which harbors the polymorphic human leukocyte antigen (HLA) class I and class II genes [4, 5]. AS is strongly associated with the HLA class I molecule B27, while RA associates with the HLA class II molecule DRB1. The propensity of this genetic background implies that the antigen presentation pathway is important to the pathogenesis of both diseases.

The initiation of all adaptive immune responses requires the display of antigenic fragments by professional antigen-presenting cells (APCs) to T cells and B cells. Considering the MHC class II antigen presentation pathway, CD74/MHC class II complexes are sorted from the endoplasmic reticulum to late endosomal compartments. Cathepsin S is an important protease in this process, cleaving the N-terminal side of CD74 to produce class II-associated invariant chain peptide (CLIP), which occludes the HLA class II peptide binding cleft and thereby prevents premature peptide loading. A defect in cathepsin S leads to the accumulation of CD74-p10, retention of the sorting signals in the N-terminus, interference with peptide loading and a block in surface-directed HLA class II transport [6-9]. Furthermore, cathepsin S deficiency shows aberrant endosomal architecture in B cells [7]. Finally, the transmembrane part of CD74 is processed by intramembrane cleavage by the protease signal peptide peptidase-like 2A (SPPL2a). Disturbed SPPL2a proteolysis triggers the accumulation of CD74-p8 fragments, enlargement of endosomes and, in contrast to cathepsin S deficiency, increases plasma membrane display of full-length CD74 and HLA class II [10-12]. The rate of CD74 proteolysis thereby imparts control over endosomal architecture and surface-directed transport of CD74 and HLA class II molecules.

IgG autoantibodies against the CD74/CLIP domain are associated with early AS [13-15]. Mechanisms contributing to this antigen-specific autoimmunity are unknown. We

considered that development of autoimmunity might be contributed by an unintentional increase in surface display of endosome residential products from CD74 by impaired SPPL2a function.

1.2 Material and methods

1.2.1. Reagents: (Z-LL_2)- ketone was purchased from Santa Cruz and recombinant IFNγ from eBioscience. The following antibodies were used: mouse anti-human LAMP-1 (BD; clone H4A3), mouse anti-human CD74 PE(Biolegend; clone LN2), mouse anti-human CD14 Pacific blue (Biolegend; clone M5E2), mouse anti-human HLA ABC PE-Cy7 (BD; clone G46-2.6), mouse anti-human HLA DR PerCP (Biolegend; clone L243), mouse anti-human CD74 N-terminal (Abcam; clone 2D1B3), goat anti- human β actin (Santa Cruz; clone I-19, goat anti-mouse AF647 (Thermofisher Scientific), rabbit anti-human Na-K ATPase (cell signalling), mouse anti-human LAMP1 (Biolegend, clone 1D4B).

1.2.2. Monocyte isolation: Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-isopaque (GE Healthcare) density gradient centrifugation. CD14+ monocytes were isolated by magnetic cell sorting using mouse anti-human CD14 magnetic beads and autoMACS Pro Separator (Miltenyi biotec) according to manufacturer's instructions.

1.2.3. Cell culture: Human monocytic leukemia cell line (THP-1) and CD14+ monocytes were cultured in RPMI-1640 (Gibco) supplemented with GlutaMAX (Fisher Scientific), 100IU/ml penicillin and 100µg/ml streptomycin, 20mM HEPES (Gibco) and 10% fetal calf serum (FCS; Biowest). Of note, cultures contained no L-glutamine, because of its known interference with endosomal acidification and CD74 processing[16]. To overcome basification of endosomes, the cells were cultured in medium containing Glutamax in all experiments. 293T human embryonic kidney (HEK293T) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat inactivated FCS, Glutamax, Penicillin and Streptomycin. Mycoplasma infection was tested and ruled on a two monthly basis.

1.2.4. Transduction of THP-1 cells using the CRISPR-Cas9 lentiviral system: A lentiviral CRISPR/Cas9 vector is used which is described previously[17]. The gRNAs were designed based on the reference human genome database. The gene-specific RNA sequences cloned was: SPPL2a KO: 5'- TGCCGGGGCCGCCCTACTCT-3'. For control KO cells we used an empty vector.

1.2.5.Lentiviral production: HEK293T cells were transfected with the CRISPR/Cas9 vector, pMD.2G, pRSV-REV, and pMDlg/p-RRE in order to produce VSVg-pseudotyped

lenti-CRISPR virions. Viral supernatants were harvested after 48h, filtered through a 0.45µm filter and used to transduce THP-1 cells by infection in the presence of 6 µg/ml polybrene. Cells were centrifuged for 1h at 700g. Transduced cells were selected with 5 µg/ml puromycin three days post transduction for 48h. The clones were validated using DNA sequencing. The sequences were analyzed using CRISPR-ID [18]. The clonally expanded cells were subjected to western blotting to test for their functional defects.

1.2.6. Protein extraction and Western blot: For the detection of CD74, $2x10^6$ monocytes were cultured with the vehicle control DMSO, 1µM $(Z\text{-}LL)_2$- ketone or 20nM LHVS for 24h at 37°C. CRISPRed THP-1 were either incubated with or without 1,000 U/ml IFNγ for 24h at 37°C. Cells were washed twice in PBS, lysed in Laemmli buffer and boiled for 5 minutes. The samples were stored at -20°C until further use. The protein concentration was determined using BCA protein assay kit (Thermo fisher Scientific). The samples were reduced using 5% β-mercaptoethanol and separated on a 4-20% polyacrylamide gradient gel (Biorad). Protein was transferred on 0.2µm PVDF-P^{SQ} (Thermo fisher scientific) membrane and blocked with 5% filtered bovine serum albumin (BSA) followed by primary antibody incubation (overnight 4°C in 0.5% BSA/TBS-T), washed 3 times in TBS-T and stained with secondary antibody (DAKO; RT, 1h in 0.5% BSA/TBS-T), washed 3 times in TBS-T and 1 times in PBS. For the detection of LAMP-1 and Na-K ATPase, endosomes were resuspended in Laemmli buffer without reducing agent and separated on 10% polyacrylamide gel. Protein was transferred on 0.45µm PVDF-P membrane and blocked with 5% dried non-fat milk. Primary and secondary antibody steps were performed as described above. The blot was developed using enhanced chemiluminescence (ECL)-prime (GE healthcare) and detected in the Bio-Rad Chemi-doc. To confirm equal protein loading, antibody against β-actin was used. The bands were quantified using ImageLab.

1.2.7. Immunofluorescence: THP-1 cells were seeded in a 24-well plate and stimulated with 1,000U/ml IFNγ for 0, 3 or 24h at 37°C. Furthermore, THP-1 cells were incubated with 20nM LHVS for 24h at 37°C. After 23.5h, 20µM Hoechst was added to each well. 8-well Lab-Tek® II Chamber Slides (ThermoFischer scientific) were pre-coated with 0.001% Poly-L-lysine for 30 minutes at RT and washed twice with PBS. The Hoechst-stained THP-1 cells were plated in the chamber slides and centrifuged for 30 seconds at 300g in order to let the cells adhere to the slides. The cells were fixed with cytofix/cytoperm (BD biosciences) for 30 minutes at RT. Cytofix/cytoperm was removed and the cells were blocked with 2% goat serum in permeabilization buffer (eBioscience) for 30 minutes at RT, followed by anti-LAMP-1 incubation (45 min, RT) and goat anti-mouse AF647 secondary antibody (ThermoFisher scientific; 45 min, RT). Between each step, the slides

were washed once with permeabilization buffer. Coverslips were mounted with Mowiol solution. All images were obtained with 1.3x optical zoom using “Plan-Apochromat” 63x 1.40 oil DIC M27 objective on a Zeiss LSM710, and processed using Zen 2009 software (Zeiss Enhanced Navigation). LAMP-1-positive endosome perimeter was analyzed using the ImageJ plug-in Squassh [19].

1.2.8. Flow cytometry: Cells were stained with the viability dye efluor® 780 (eBioscience) in PBS for 30 min at 4°C, washed in FACS buffer (PBS supplemented with 1% BSA and 0.1% sodium azide), stained for antibodies directed against CD14, CD74, CLIP, HLA-DR and HLA-ABC plus 10% mouse serum for 15 min at 4°C. Cells were washed and acquired on BD Fortessa flow cytometer. Analysis was done with FlowJo.

1.2.9. Endosome and plasma membrane isolation by percoll density gradient centrifugation: Endosome and plasma membrane isolation was performed as described previously with minor modifications [20]**.** Briefly, $50*10^6$ THP-1 cells were incubated with 500U/ml IFNγ for 24h at 37°C, harvested and washed twice in PBS. Cells were resuspended in Homogenization Buffer (HB; 250 mM sucrose, 25 mM Tris-HCl pH 7.4 and 1 mM EDTA and complete protease inhibitor) and disrupted mechanically by 5 passages through a 25 gauge syringe needle. A centrifugation step of 1,000g for 10 minutes was performed to obtain a post-nuclear supernatant. In a 13 ml tube, 8.7 ml of 20% Percoll solution in HB was underlayered with 0.5ml of 65% (w/v) sucrose in Tris-HCl, pH7.4. On top, 1 ml of post-nuclear supernatant was added. Finally, the tubes were topped off to a volume of 12 ml with PBS. Centrifugation was performed in centrifuge Optima L-90K and LE-80K (Beckman Coulter Optima) in SW40 Ti rotor at 40,500g for 60 minutes at 4°C. Fractions of 500µl were collected from top (fraction 1) to bottom (fraction 24). The fractions were topped off to a volume of 5 ml with PBS and centrifuged in a SW60 Ti rotor (Beckman Coulter) at 100,000g for 1h5min. The pellet was resuspended in Laemmli buffer for western blot purposes.

1.2.10. Immunoprecipitation: $5x10^6$ THP-1 cells were cultured with 500U/ml IFNγ for 24h at 37°C , washed and lysed in CHAPS buffer (1% CHAPS, 30 mM Tris-HCL pH 8.0, 150 mM NaCl) for 45 minutes on ice and spun down at 13,000g for 15 minutes. Supernatant was cleared with beads and incubated with beads coupled to antibodies that were present in human serum (Protein G-agarose beads, Roche) for 16 hours under constant agitation. Beads were separated from lysates by centrifugation, washed twice CHAPS buffer and resuspended in Laemmli buffer. The sample was boiled and protein interactions were analyzed using Western blotting (see above).

1.2.11 Statistics: Cohorts of healthy donors and patients investigated for CD74-p8 accumulation and cell surface marker expression were analyzed using two-tailed unpaired t tests. Confocal microscopy results were analyzed using one-way ANOVA, followed by a Tukey-Kramer test. Biological replicates for each data point are included in the figure legends. Statistical analyses were performed using Prism 7 (GraphPad Software Inc.).

1.3. Results

1.3.1. Monocytes from AS patients have decreased SPPL2a enzyme activity.

We first took a pharmacologic inhibition approach by culturing monocytes in the presence or absence of the SPP/SPPL inhibitor $(Z\text{-}LL)_2$-ketone for 24 hours (IC50 dose, 1uM [21-23]). CD74-p8 accumulation was tested on Western blot. As a control, we included lysates from monocytes, derived from healthy control (HC) donors, that were treated with cathepsin S inhibitor LHVS (20nM). LHVS blocks the endosomal proteolysis of CD74-p10 to CLIP [6]. Monocytes were still viable at the concentrations $(Z\text{-}LL)_2$-ketone and LHVS used (data not shown). Confirming earlier results, the SPPL2a inhibitor-treated monocytes showed N-terminal fraction (NTF) accumulation of 8 kDa (CD74-p8) [10-12], and the cathepsin S inhibitor-treated monocytes revealed accumulation of CD74-p10 **(Fig. 1A)**. Thus, both enzymes function in processing of CD74 in human monocytes.

We next examined the SPPL2a enzyme function in monocytes from HC donors and patients diagnosed with PsA, RA and AS (**Table 1**). Lysates from monocytes were tested for CD74-p8 accumulation on Western blot. A few patients showed CD74-p8 accumulation at baseline **(Fig. 1B)**. To increase the experimental window in order to measure SPPL2a enzyme activity, monocytes from HC and patients were also treated with 1μM $(Z\text{-}LL)_2$ ketone. We first quantified and normalized the full-length of CD74 (33/35kDa) in each sample to β-actin and found that $(Z\text{-}LL)_2$-treated monocytes from both RA and AS patients showed significantly higher CD74-p33/35 accumulation compared to HC **(Fig. S1A)**. To exclude that the accumulation of CD74-p8 is due to increased substrate availability, CD74-p8 was normalized to full-length CD74. After full-length CD74 correction, monocytes from AS patients showed significantly more CD74-p8 accumulation upon SPPL2a inhibition compared to HC (**Fig. 1C**). Thus, CD74 proteolysis by SPPL2a is affected in monocytes from AS patients.

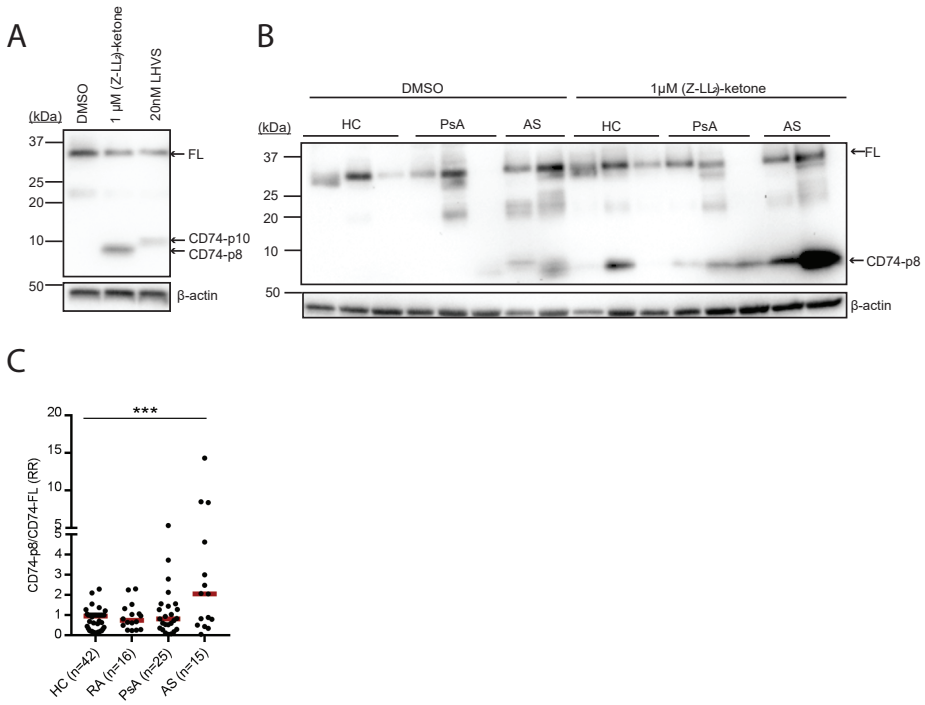


Fig. 1. Monocytes from AS patients show an increase in CD74-p8 accumulation.
(**A** to **C**) CD14+ monocytes were isolated from PBMCs from HC, RA, PsA and AS by CD14 magnetic beads (**A** to **B**). CD14+ monocytes were incubated with the vehicle control DMSO, 1μM $(Z\text{-}LL)_2$ ketone or 20nM LHVS for 24h. At least 2 HCs were included on each Western blot. The protein lysates were tested for CD74 using an antibody directed against de N-terminus (clone: 2D1B3). (**C)** The blot was stripped and reprobed for β-actin as a loading control (Sixteen independent experiments). CD74-p8 was normalized to CD74-p33/p35 following normalization to a HC. The mean is represented in the dot plot ***p<0.001; unpaired *t* test.

Table 1: Clinical and demographic features

	HC	RA	PsA	AS
Number of patients	47	16	27	18
Age (median (range))	45 (23-65)	61 (26-73)	54 (31-73)	42 (19-65)
Female	49%	75%	22%	28%
Duration disease in years (median (range))	n.a.	10 (1-28)	11 (1-28)	9 (0.5-30)
Age disease-onset (median (range))	n.a.	46 (14-69)	44 (24-66)	33 (13-57)
HLA-B27+	n.a.	n.a.	n.a.	83%
RF+	n.a.	69%	4%	0%
axial involvement	n.a.	0%	4%	100%
CRP (median (range))	n.a.	6.4 (1.1-8)	2.9 (0,5-4.9)	10 (0.5-45)
ESR (median (range))	n.a.	7 (5-47)	8 (1-42)	13 (1-42)
TJC (median (range))	n.a.	4 (0-13)	1 (0-26)	0 (0-4)
SJC (median (range))	n.a.	3 (0-13)	0 (0-13)	0 (0-4)
Systemic immunomodulatory medication	n.a.	100%	74%	17%

1.3.2. Knockout of SPPL2a results in retention of CD74-p8 in response to IFNγ.

To understand the function of SPPL2a in monocytes, we created SPPL2a-deficient cells using CRISPR-based whole genome editing in a human monocytic cell line THP-1. Additionally, we considered that interferon gamma (IFNγ) plays an important role in the antigen presentation pathway and CD74 processing [24-26], and therefore tested CD74-p8 accumulation in SPPL2a wildtype and SPPL2a knock out (KO) THP-1 cells, in both the absence and presence of IFNγ. We found that both untreated and IFNγ-treated SPPL2a KO cells yield CD74 accumulation **(Fig. 2A, B)**. However, IFNγ-treated SPPL2a KO cells showed a higher expression of CD74-p8. In order to confirm that the NTF retained in IFNγ-stimulated SPPL2a KO cells corresponds to CD74-p8, we compared the NTF with $(Z\text{-}LL)_2$-ketone-treated THP-1 cells. The size of the accumulated in SPPL2a cells resembled that of the accumulated NTF in SPPL2a-inhibited THP-1 cells, confirming the SPPL2a KO. The SPPL2a KO cells are further confirmed by DNA sequencing **(Fig. S2A)**. This data suggest that the inflammatory signal IFNγ increases CD74-p8 accumulation in THP-1 cells and we continued our experiments using IFNγ-stimulated SPPL2a KO THP-1 cells.

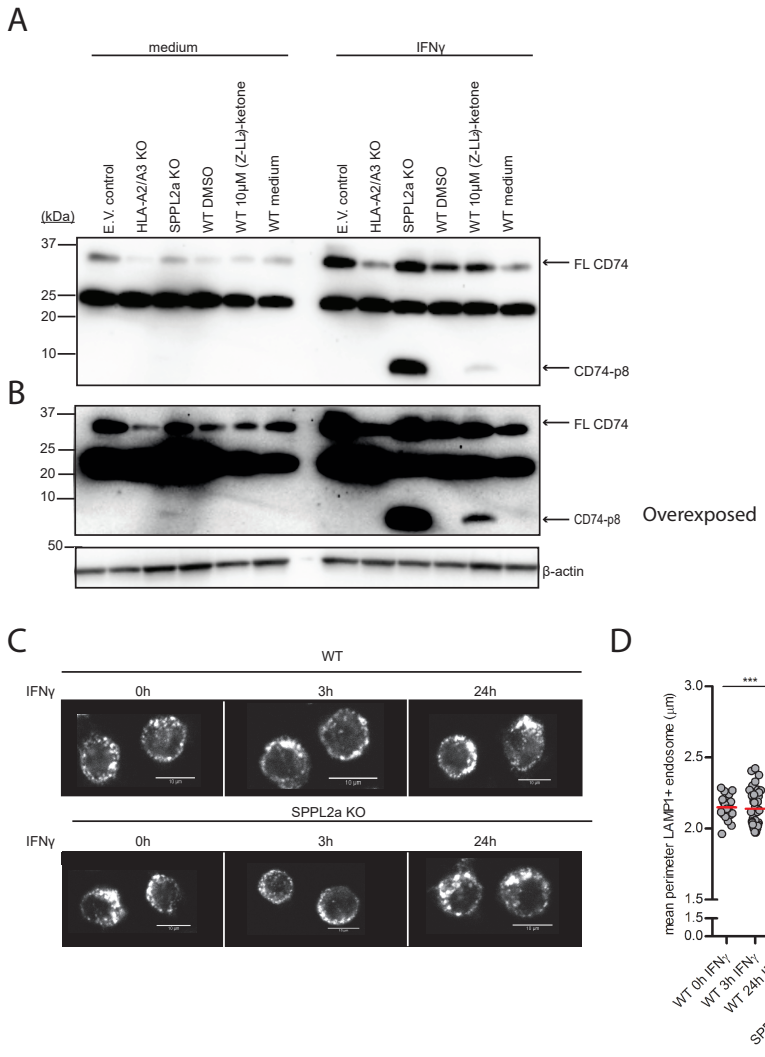


Fig. 2. SPPL2a KO cells show compression in endosomal perimeter size upon IFNγ stimulation. For the generation of a SPPL2a KO cell line, THP-1 was depleted for the targets using CRISPR/Cas9 combined with lentiviral transduction technology. Empty vector was used as a CRISPR control. (**A**) The effect of CRISPR gene destruction in THP-1 cells was evaluated on western blot. The CRISPRed cells were treated with 1,000 U/ml IFNγ for 24h. WT cells were treated with vehicle control DMSO or 1μM $(Z\text{-}LL)_2$-ketone in the presence or absence of 1,000 U/ml IFNγ for 24h. Western blot lysates were blotted for CD74 N-terminus for the detection of full length (FL) CD74 and CD74 NTF. Representative of three independent experiments. (**B**) Representative immunofluorescence images of LAMP-1+ endosomes of WT and SPPL2a KO THP-1 cells that were treated with or without 1,000 U/ml IFNγ for 0, 3 or 24 hours. Scale bars: 10μm.

Fig.2. Continued

(**C**) Dot plot represents quantification of the perimeter of LAMP-1+ endosomes. Eight to nine pictures with at least 20 cells from each condition were taken. Each dot represents the mean endosomal LAMP1+ perimeter from one confocal image, analyzed by the Image J plug-in Squassh. The dot plot shows the mean (n=4-5 from five independent experiments). ***p<0.001; **** p<0.0001; one-way ANOVA, Tukey's test.

1.3.3. SPPL2A KO THP-1 cells show compressed LAMP-1+ endosomes in response to IFNγ.

To test if endosomal accumulation of CD74-p8 drive morphological changes of late endosomes, we measured the average size of endosomes of SPPL2a KO and WT THP-1 cells. We included IFNγ stimulation as a prerequisite condition to examine SPPL2a dysfunction on endosomal morphology in THP-1 cells. While both WT and E.V. control THP1 cells showed similarly sized endosomes in the steady state, IFNγ-stimulation triggered endosomal enlargement after 24h **(Fig. S3A, B)**. Pre-treatment with cathepsin S inhibitor LHVS resulted in endosomal enlargement [7], which did not enlarge further upon IFNγ stimulation. Similarly, SPPL2a KO cells revealed enlarged endosomes at basal level [12] with no additional changes induced after 24h IFNγ stimulation **(Fig. S3A, B)**. However, SPPL2a KO cells showed compressed endosomes after 3h IFNγ stimulation that return to the original size by 24h, which is not observed in WT cells (**Fig 2C, D)**. Overall, these data suggest that SPPL2a KO cells can resolve the endosomally accumulated CD74 fragments by IFNγ-induced traffic to the surface, after which endosomes reverse to basal level.

1.3.4. SPPL2A KO THP-1 cells exhibit increased surface display of peptide-bound HLA-DR, full-length CD74 in response to IFNγ.

To test if SPPL2a deficiency might trigger increased plasma membrane expression of CD74 and possibly other endosome-derived molecules, we stimulated THP-1 cells with IFNγ and assessed the cell surface expression of full-length CD74, peptide-bound HLA-DR, CLIP-bound HLA-DR and HLA-ABC by flow cytometry. Under unstimulated conditions, we observed low levels of CLIP, HLA-DR, full-length CD74 and HLA-ABC in WT, E.V. control and SPPL2a KO THP-1 **(Fig. 3A, B)**. Upon 24h IFNγ treatment, SPPL2a KO THP-1 cells showed significant increased expression of CD74, HLA-DR and CLIP-bound HLA-DR compared to controls **(Fig. 3A, B)**. We observed no difference in HLA-ABC surface expression between IFNγ-exposed control cells and SPPL2a deficient cells, confirming that IFNγ-induced HLA-ABC surface display does not contribute significantly to late endosomal stores [27]. Therefore, the compressed endosomal perimeter in SPPL2a KO associates with increased surface-directed transport of full-length CD74, HLA-DR and CLIP-bound HLA-DR.

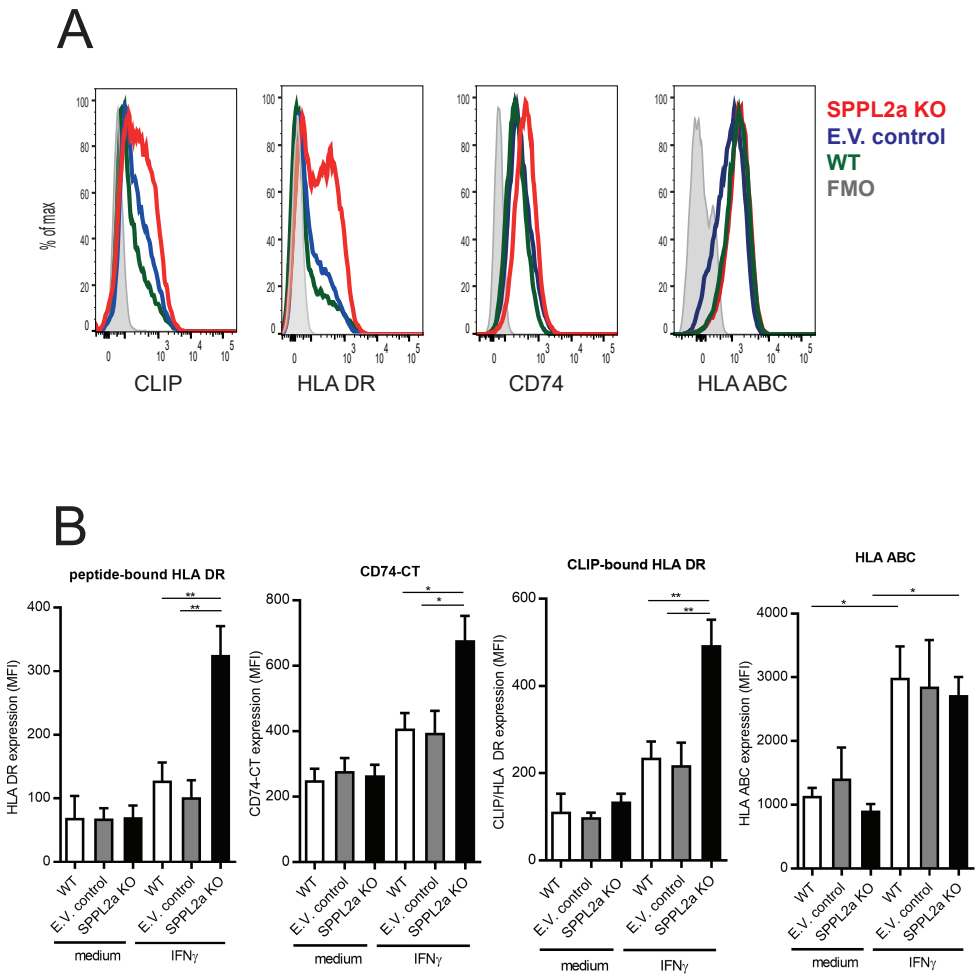


Fig. 3. SPPL2a KO cells exhibit increased surface display of full-length CD74, CLIP-bound HLA-DR, peptide-bound HLA-DR and CD74-p8 expression upon IFNγ stimulation.
(A) Representative histogram of flow cytometry analysis of expression of full-length CD74 C-terminal (LN2), CLIP-bound to HLA-DR (CerCLIP), peptide-bound HLA-DR (L243) and HLA-ABC (G46-2.6) on THP-1 incubated in the presence of IFNγ for 24h was assessed by flow cytometry. (**B**) Error bars show the mean fluorescence intensity (MFI) and S.E.M. from four independent experiments. *p<0.05; **p<0.01; unpaired *t* test.

1.3.5. Monocytes from AS patients have increased expression of full-length CD74 and HLA-DR.

We asked whether defective SPPL2A function in monocytes from patients with AS and PsA relate to increased cell surface markers. We observed a significant accumulation of full-length CD74 and peptide-bound HLA-DR on the cell surface of AS monocytes, but not in PsA (**Fig. 4A, B**). CLIP-bound HLA-DR and HLA-ABC were similarly expressed throughout

all individuals investigated **(Fig. 4A, B)**. Thus, monocytes from AS patients were more prone to increased cell surface expression of HLA class II and full-length CD74.

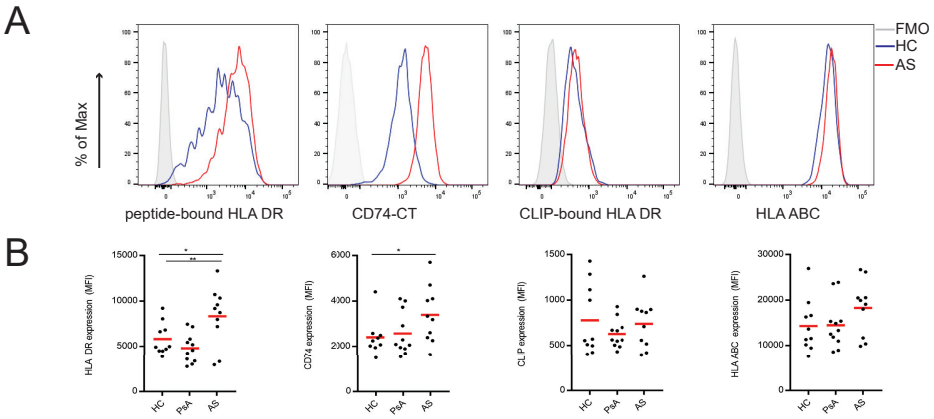


Fig. 4. Monocytes from AS patients have increased full-length CD74 and peptide-bound HLA-DR expression

(**A** to **B**) Expression of full-length CD74, CLIP-bound to HLA-DR, peptide-bound to HLA-DR and HLA-ABC on monocytes from PBMCs from HC, PsA and AS were analyzed using flow cytometry. (**A**) Representative flow cytometry data is shown in histograms. Grey histogram represents fluorescence minus one (FMO) control, blue line represents a HC, red line represents an AS patient. (B) Dot plot represents the mean fluorescence intensity (MFI) of the cell surface molecules measured from each donor. The mean is represented in the dot plot (three independent experiments). *p<0.05; **p<0.01; unpaired *t* test.

1.3.6. IFNγ treatment triggers increased surface display of CD74-p8 N-terminal fragment in SPPL2a KO THP1 cells.

To investigate whether NTF (CD74-p8) can also accumulate on the plasma membrane, we stimulated THP-1 cells with IFNγ for 24h and isolated subcellular fractions by discontinuous Percoll-sucrose density gradient, which we thereafter subjected to Western blot. As positive controls, whole lysates showed expression of LAMP1, Na+/K+ ATPase, and both full-length CD74 and CD74-p8 (two lanes on the right side of **Fig. 5A and B**). Both E.V. control and SPPL2a KO cells showed co-localization of full-length CD74 with the plasma membrane marker Na^+/K^+ ATPase **(Fig. 5A)**. E.V. control did not reveal CD74-p8 accumulation. However, in SPPL2a KO, CD74-p8 was enriched in both the LAMP-1 fraction, late endosomes (fraction 22, **Fig. 5B**), and the Na^+/K^+ ATPase and LAMP-1, cell surface fraction (fraction 8-10 **Fig. 5A**). It is known that LAMP-1 is present in transport vesicles, directed to fuse with the plasma membrane[28]. These data suggest that NTF (CD74-p8) is transported to the plasma membrane from the late endosomes/lysosomes.

1.3.7. Sera from AS patients contain IgG antibodies directed against CD74 fragments that accumulate in SPPL2a KO THP-1 cells.

We finally investigated whether sera from AS patients contain IgG antibodies directed against CD74 fragments and performed an antibody-protein pull down experiment. We selected sera from four AS patients who showed high NTF (CD74-p8) accumulation, and 1 HC as control. We used lysates from IFNγ-treated E.V. control and IFNγ-treated SPPL2a KO THP-1 cells as a source of CD74 protein fragments, and screened for the presence of anti-CD74 antibodies by preincubation of protein G agarose beads with patient or control serum. Immunoprecipitated CD74 fragments, as measure for the presence of anti-CD74 antibodies, were examined using Western blot. As expected, CD74 fragments were not detected in E.V. control lysates (**Fig. 5C**). However, we observed IgG antibodies in serum of 3 AS patients, as these patient sera detected CD74 fragments in lysates from SPPl2a KO cells (**Fig. 5C**). Antibodies that were present in serum samples from HC and one AS patient did not detect CD74 fragments. The molecular weight of the detected CD74 fragments is higher compared to CD74-p8. This corroborate the finding that auto-antibodies recognize CLIP, which is not present in CD74-p8 [13, 29]. Together, these data support that decreases in SPPL2a function contributes to a change in subcellular location of CD74 fragments, which adds to the development of antigen-specific autoimmunity.

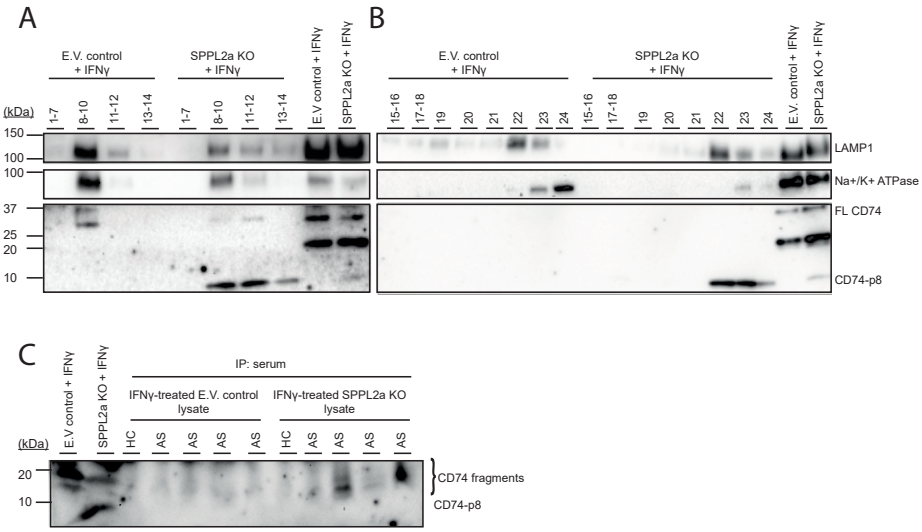


Fig. 5. Sera from AS patients contain IgG CD74-autoantibodies.

Fig. 5. Continued
(**A** en **B**) E.V. control and SPPL2a KO THP-1 cells were stimulated with 1,000 U/ml IFNγ for 24h and lysed in HB buffer and subcellularly fractionated using a Percoll-density gradient. Twelve fractions were collected with a volume of 500 µl (top fraction 1, bottom fraction 24). Fractions 1-7, 8-10, 11-12, 13-14, 15-16, 17-18, 19-20, 21, 22, 23, 24 were collected and centrifuged to pellet the organelles and lysed in Laemmli buffer and analyzed for LAMP-1, CD74 N-terminus, and Na^+/K^+ ATPase expression using Western blot (n=2 from two independent experiments). (**C**) protein-G agarose beads were coated with serum from AS patients and HCs. IFNγ-treated E.V. control and SPPL2a KO THP-1 cells were lysated and proteins were immunoprecipitated using serum-coated beads. Immunoprecipitates were examined using Western blot (n=2 from two independent experiments).

1.4. Discussion

In this study, we considered that the development of anti-CD74 autoantibodies might be contributed by an unintentional increase in surface display of endosome residential products, most notably CD74. We investigated the potential role of the late endosomal protease SPPL2a and its substrate CD74 in AS. We found clear defects in SPPL2a function and CD74 processing in AS patients, which culminated into increased CD74 and HLA class II display at the cell surface, and production of IgG autoantibodies specific to CD74. These findings are corroborated by the observations in SPPL2a deficient THP-1 cells, which showed compressed late endosomes upon receipt of an inflammatory trigger. A feature that coincided with increased expression of MHC class II and total CD74 at the plasma membrane. These data reveal a clear distinction between SPPL2a and cathepsin S, as in cathepsin S KO cells the MHC class II molecule expression on the cell surface is impeded [7, 10-12]. These results support that cathepsin S proteolysis facilitates MHC class II transport, whereas SPPL2a proteolysis prevents premature MHC class II surface-directed transport.

The observation that a defect in SPPL2a leads to enlarged endosomes and, upon inflammation, to compressed endosomes indicates that CD74 directs the fusion and retention mechanisms of the endosomal pathway[30-32]. Indeed, a double knock-out for both SPPL2a and CD74 prevents accumulation of late endosomal multivesicular bodies (MVBs) in mouse B cells[12]. The mechanisms whereby CD74 control endosomal size is, for example, via the interaction between the NTF of CD74 and 70-kDa heat-shock cognate protein [33]. Also the free negative charges in the N-terminus are involved in endosome fusion to form MVBs[34]. While in MVBs, CD74 is efficiently proteolysed by cysteine proteases, during which cathepsin S cleaves Ii-p10 into CLIP, allowing the loading of new peptide-receptive MHC class II molecules[20, 35]. The CD74-p8 substrate for SPPL2a contains the CD74 N-terminal cytoplasmic region. Thus, the aberration in endosomal morphology as a consequence to SPPL2a deficiency is caused by the

accumulation of free NTF chains through interference with endosomal fusion and retention of membrane proteins.

To our knowledge, this is the first study to describe the increased display of CD74-p8 at the plasma membrane in response to a defect in SPPL2a activity and upon exposure to IFNγ. The main binding site of CD74 to MHC class II is via the CLIP domain[36]. Besides through CLIP binding, the transmembrane region of CD74 can also weakly bind to MHC class II[37]. Therefore, a fraction of the accumulated CD74-p8 in SPPL2a deficient cells, which is a cleaved fragment separate from CLIP, might still be attached to MHC class II. Based on this assertion, the trafficking of MHC class II molecules to the plasma membrane can be accompanied by CD74-p8. The accumulation of CD74-p8 fragments at the plasma membrane, which under healthy circumstances remains mostly hidden from humoral recognition, might trigger an immune response towards autoantibody production to CD74 in AS patients. Indeed, we found that monocytes from patients incubated with a SPP/SPPL inhibitor revealed an increase in CD74-p8 accumulation and the presence of IgG anti-CD74 autoantibodies. In addition, it is unclear why monocytes from PsA did not reveal a significant SPPL2a dysfunction and the presence of anti-CLIP antibodies, considering that PsA shares some immunologic and phenotypic overlap with AS. Important to note in this respect is the heterogeneity in SPPL2a dysfunction seen in monocytes from AS patients, which supports that causation of the disease and/or the chronic inflammatory state are not singular, and might evolve during the course of the disease.

A limitation of our study is the technical challenge to detect IgG anti-CD74 antibodies in the serum of AS patients by ELISA, which we attribute to the incompatibility of detergents we used to generate THP-1 cell lysates as source of CD74 protein fragments, with the antibodies and substrates for ELISA. For this reason, we resorted to Western blot-based detection, which allowed for the repeated detection of anti-CD74 reactivity in AS sera and not control sera.

Recently, a potential oral bioavailable inhibitor has been described to significantly and selectively inhibit SPPL2a in mice and rats and as a consequence resulted in a decrease in B cell and mDC numbers [38, 39]. SPPPL2a inhibition may suppress inflammation, but with the new knowledge that the inhibition of SPPL2a can break immune tolerance towards CD74, can also worsen autoimmunity.

In summary, our data supports that a dysfunction in the protease SPPL2a leads to dysregulation of CD74 processing. Accordingly, exposure to inflammatory stimuli

triggers the surface-directed transport of late endosomal cargo for increased display at the plasma membrane of CD74/CLIP. We propose that such increase in display may provoke the immune system to produce autoantibodies to CD74, in analogy to viral particles that are kept hidden from the humoral immune system until displayed at the cell surface. Our research for the first time provides a mechanistic explanation for the CLIP domain-specific IgG that is seen in 85% of AS patients [29].

Acknowledgements
We thank Lotte Spel, Sandra Silva-Cardoso, Willemijn Janssen and Toine ten Broeke for their feedback and discussions; Vincent Verwijmeren for computerizing the software Squassh, Prof. Dr. W. Stoorvogel, Utrecht University, for using the ultracentrifuge facility; Prof. Dr. L. Meyaard, University Medical Center, Utrecht, for sharing the lentiviral vectors; the Flow Cytometry Core Facility for their assistance; Mini donor service for their help in collecting healthy control blood samples.

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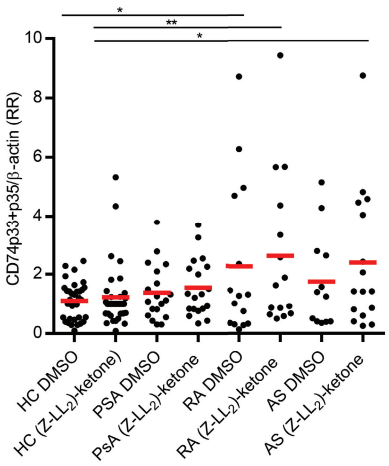


Fig. S1. Full-length CD74 protein expression quantification
(**A**) CD14+ monocytes were isolated from PBMCs from HC, RA, PsA and AS by CD14 magnetic cell sorter beads. CD14+ monocytes were incubated with the vehicle control DMSO or 1μM $(Z\text{-}LL)_2$ for 24h. At least 2 HCs were included on each western blot. The protein lysates were analyzed for the presence of CD74 protein using an antibody directed against de N-terminus (clone: 2D1B3). The blot was stripped and reprobed for β-actin as a loading control (Sixteen independent experiments). CD74 full length was normalized to β-actin following normalization to a HC. The mean is represented in the dot plot *p<0.05; **p<0.01; unpaired *t* test.

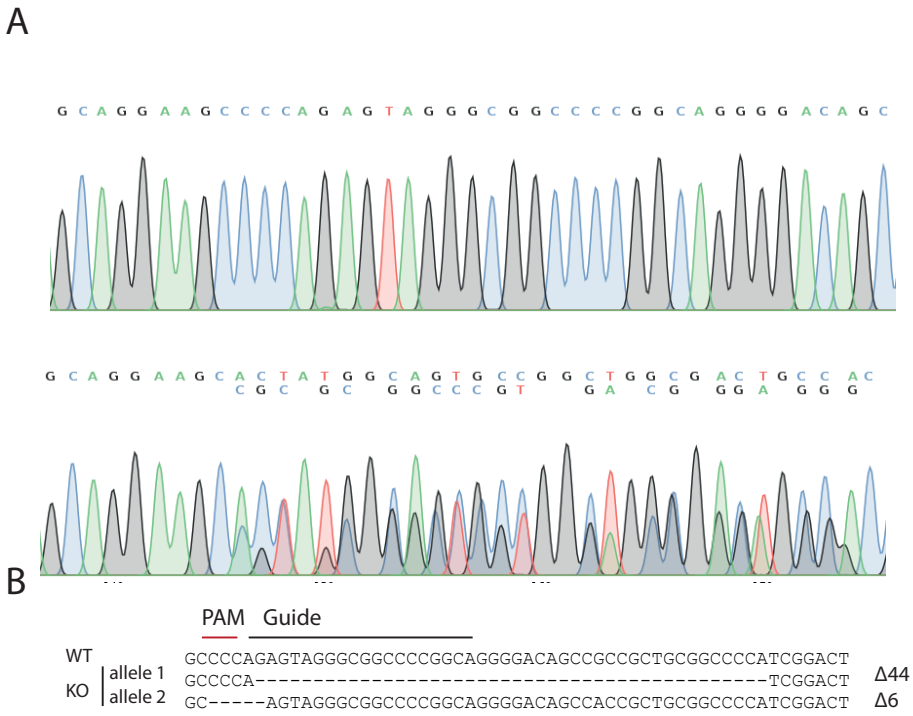


Fig. S2. Sequence analysis of SPPL2a KO cells
Genomic DNA was amplified by PCR and subjected to sequencing. (**A**) representative sequencing images of WT and SPPL2a KO cells upon CRISPR/Cas9 targeting. The gRNA-target site and PAM sequence is underlined in the figure. (**B**) Sequence variants of both alleles are depicted.

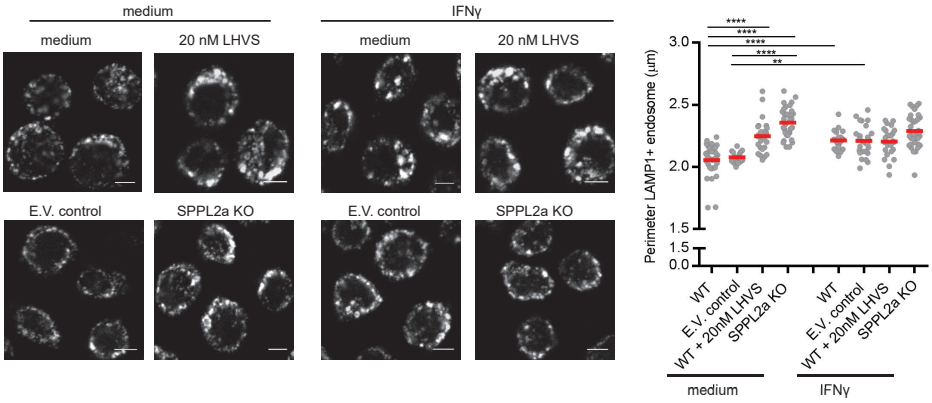
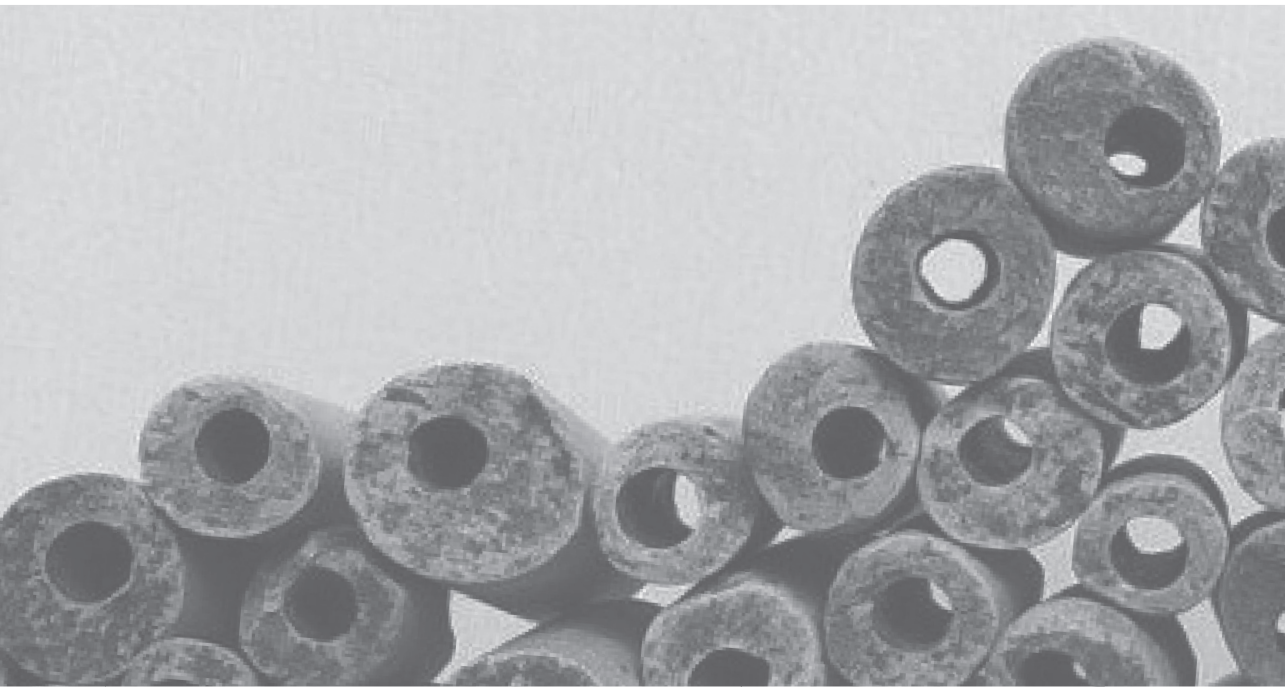
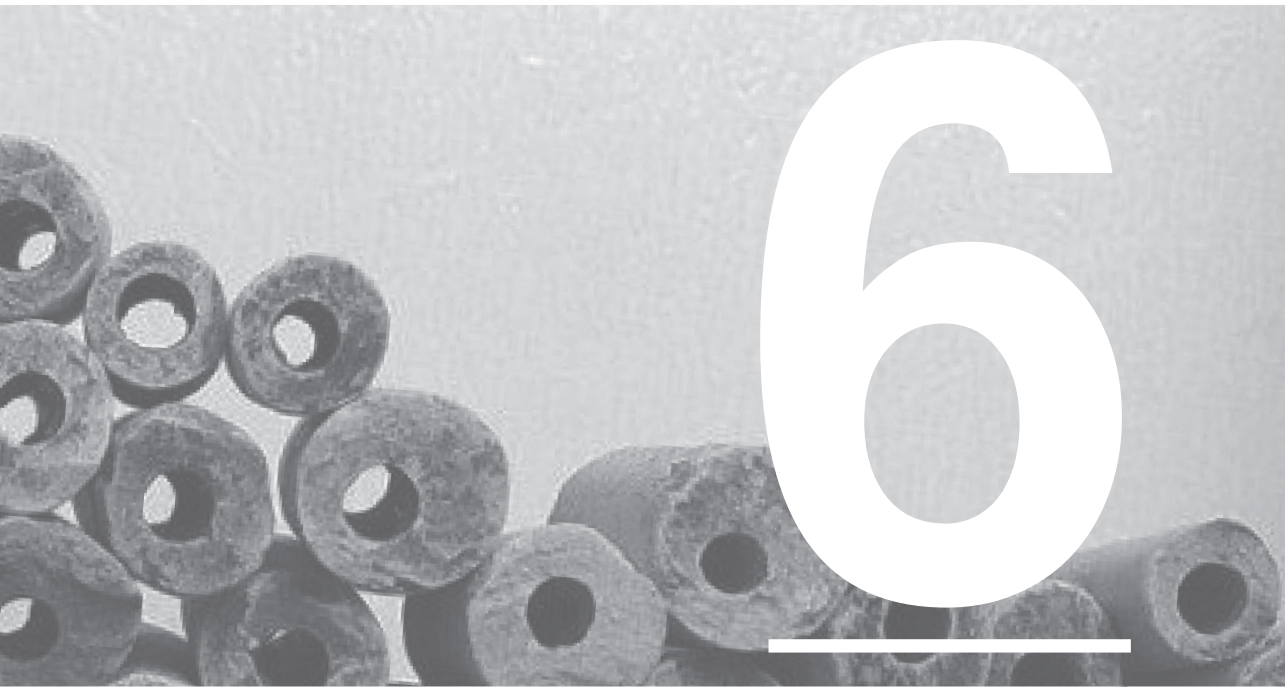


Fig. S3. SPPL2a KO cells have enlarged late endosomes at baseline.
(**A**) Representative immunofluorescence images of LAMP-1+ endosomes of WT, E.V. control and SPPL2a KO THP-1 cells that were treated with or without 1,000 U/ml IFNγ or with 20 nM LHVS. Scale bars: 10µm. (**B**) Dot plot represents quantification of the perimeter of LAMP-1+ endosomes. Eight to nine pictures with at least 20 cells from each condition were taken. Each dot represents the mean endosomal LAMP1+ perimeter from one confocal image, analyzed by the Image J plug-in Squassh. The dot plot shows the mean (n=4-5 from five independent experiments). *p<0.05; **p<0.01;*** p<0.001; **** p<0.0001; one-way ANOVA, Tukey's test.





6

General discussion

Our health relies on an effective immune system. Although many mechanistic elements of adaptive immunity have been resolved during the last decades, our knowledge of its complexity is still far from complete. Fundamental research on molecular and cellular aspects of the immune system has contributed to the development and application of novel immune therapies. Nevertheless, such therapies are still largely developed on trial and error basis, and their efficacies are often disappointing. For example, while vaccines in general have contributed immensely to both human and veterinary health, we still do not fully understand precisely how foreign antigens are being discriminated from "self" in adoptive immune responses, leaving challenges for designing vaccines that are more effective and safe. Although the essential role for dendritic cells (DCs) for initiating adaptive immune responses has been well established, the intricacies of how DCs discriminate between presentation of pathogen-derived peptides versus self-peptides remain unclear. We propose that exosomes from dendritic cells may play a decisive role in this respect. In this thesis we started to explore the hypothesis that DC-derived exosomes, as a mechanism employed by our immune-system, may selectively present peptides derived from phagocytosed pathogens. As a result, the risk of undesired presentation of self-peptides with the potential consequence of developing autoimmunity could be reduced. Previously, it has already been demonstrated that DC-derived extracellular vesicles (EVs) carry peptide loaded MHC class I and II complexes with a capacity to activate naïve T-cells[1–6]. However, the exact triggers and mechanisms underlying the release of these EVs, as well as the significance of their contribution for activation of the immune system at physiological conditions, remain ill-defined. In this thesis we explore different triggers that induce the release of EVs by DCs and studied how these triggers may contribute to their function.

Self- versus non-self antigen presentation

Immature DCs only present self-peptides on MHC class I and II. Contact with pathogens induces the DC to mature, enabling presentation of pathogen-derived peptides on MHC class I and II. At the same time, activation of DCs induces their migration towards lymphoid organs as well as the expression of co-stimulatory molecules and immune-stimulatory cytokines, providing the conditions to activate naïve cognate T-cells[7,8]. While only a few hundred copies of specific MHC-antigen complexes on activated DCs are sufficient to activate T-cells[9,10], activated DCs simultaneously display millions of peptides originating from self-proteins rather than from pathogens[11]. This poses an important conceptual problem: How does the immune system prevent activation of self-reactive T-cells by activated DC that in addition to foreign peptides also abundantly display self-peptides in the context of MHC?

T-cell tolerance towards self is generated at multiple levels: 1, T-cells carrying a T-cell receptor (TCR) with high affinity for self-peptide–MHC complexes are deleted by thymic selection mechanisms. 2, T-cells can be eliminated through induction of T-cell anergy or by regulatory T-cells (Treg)[12–14]. 3, Several mechanisms in DCs favor presentation of pathogen-derived peptides over peptides from self-proteins. With respect to the latter, firstly, the lumen of phagosomes is only slightly acidified in DCs, resulting in limited proteolytic degradation of phagocytosed pathogens and preventing complete degradation of generated peptides. In contrast, self-peptides generated in fully acidified endosomes and lysosomes are more readily degraded and thus less available for loading onto MHC molecules[15]. Secondly, each phagosome can signal and mature autonomously, resulting in a distinct fate of MHC molecules from pathogen-containing phagosomes[16–19]. For example, TLR4-activation in phagosomes results in the recruitment of MHC class I molecules to phagosomes, increasing the cross-presentation of pathogen-derived peptides on the plasma membrane[20]. Similarly, phagosomal TLR-triggering favors selective antigen presentation by MHC class II from phagosomes[21]. Nevertheless, as indicated above, by far most MHC molecules expressed on the plasma membrane present "self" peptides. Thus, a potentially dangerous situation is created, as the presentation of self-peptides in combination with costimulatory molecules on activated DCs might result in the activation of remaining autoreactive T-cells. Indeed self-reactivity is not effectively prevented in all circumstances, as exemplified by the occurrence of autoimmune diseases.

DC-derived EVs can carry peptide-MHC complexes as well as costimulatory molecules, and have been shown to be capable of inducing T-cell activation either directly or via bystander DCs[2,3,22]. Therefore, we propose that DC-derived EVs may function in an additional layer of regulation, favoring presentation of pathogen-derived peptides relative to self-peptides, thus preventing activation of auto-reactive T-cells.

Extracellular vesicles

The term "extracellular vesicles" encompasses microvesicles (MVs), which bud directly from the plasma membrane, and exosomes, which originate as intraluminal vesicles (ILVs) in multivesicular bodies (MVBs) and are released upon fusion of MVBs with the plasma membrane. Due to their overlapping biophysical characteristics, absolute segregation of MVs and exosomes is not possible using current technologies[23]. However, for experimental analysis, EVs are often divided in two subsets by use of sequential centrifugation steps: larger MVs predominantly pellet at 10,000 x g, and exosomes are enriched in 100,000 x g pellets[23,24]. When collected from cultured immature DCs, these

two isolated EV-populations had distinct effects on T-cell differentiation, with large EVs predominantly inducing Th2 (humoral) responses, and small EVs stimulating Th1 (cell-mediated) responses[25]. It should be noted, however, that cultured immature DCs are often contaminated with activated DCs. The maturation status of the DCs also plays a role: when isolated from IFN-γ activated DCs, EVs from 10,000 x g and 100,000 x g pellets induced similar T-cell-activating responses[25]. We restricted our experiments to EVs released by mature DCs, as only these EVs effectively activate naïve MHC-peptide restricted cognate T-cells[2,3].

Bystander T-cells induce the release by DCs of antigen presenting EVs

DC-functionality is shaped by the combined actions of innate and adaptive signals. For example, when antigen-specific CD4+ T-cells interact with activated DCs carrying cognate MHC-II/peptide complexes, DCs increase their expression of CD40L, licensing the DCs to stimulate CD8+ T-cell responses[26,27]. However, activated CD4+ T-cells can also promote maturation of bystander dendritic cells, driving subsequent activation of cognate T-cells by these DCs[28]. It is not well-understood how bystander T-cells improve the efficiency with which DCs activate cognate T-cells. Previously, we found that antigen-specific T-cells promoted the release of EVs by antigen-loaded cocultured DCs[5]. Such antigen-dependent interactions provide maturation stimuli for both DCs and T cells, and it was therefore not clear whether the cognate interaction itself or the state of maturation of the cells was responsible for the increase in EV release. We hypothesized that activated bystander T-cells might induce the release of more, or different, EVs by matured DCs when compared to naïve T-cells. In **chapter 3**, we show that non-cognate interactions between activated T-cells and LPS-experienced DCs induced the DCs to adopt a more immune-stimulatory phenotype: MHC class II expression was increased and protrusions from the plasma membrane were extended. Importantly, we found that non-cognate interactions between LPS-activated DCs and activated T-cells stimulated the release of DC-derived EVs containing MHC class I, CD63 and ICAM-1, as well as an immune stimulatory miRNA, miR155. Upon loading of a CMV peptide on EV-resident MHC class I, , they could activate CMV-specific T-cells. Based on these data, we propose that pathogen-experienced DCs traveling to lymphoid tissues are stimulated by activated bystander T-cells to locally release EVs into the appropriate environment. These EVs are enriched for molecules that can stimulate antigen specific T-cells, including MHC-complexes containing pathogen-derived peptides, as well as co-stimulatory molecules, integrins, and immune-modulatory RNA-species. Activation of T-cells by EVs may occur either directly or upon recruitment of the EVs by bystander DCs, ultimately facilitating cognate T-cell activation. Thus, the pre-existing balance of

activated and non-activated bystander T-cells in the lymph node helps shaping immune responses via DC-derived EVs, while dissemination of MHC-peptide complexes via EVs themselves, or via EVs to bystander DCs could increase the chance of, or amplify, activation of antigen-specific T cells.

DCs release exosomes concomitantly with phagocytosed pathogens

Macrophages and epithelial cells have been demonstrated to release phagocytosed pathogens through non-lytic expulsion, involving direct fusion of the phagosome with the plasma membrane[29–35]. Expulsion of pathogens from the phagosome has been shown to occur upon intracellular TLR4 signaling[36,37] and EVs may be co- released in the process[36]. In **chapter 4** we demonstrate for the first time that in response to being challenged by *E.coli*, DCs are also capable of secreting a significant proportion of previously phagocytosed *E.coli*. Moreover, we show that expulsion of *E.coli* coincides with the release of exosomes from these phagosomes. These findings suggest that MHC molecules present on exosomes may be selectively loaded with antigens derived from phagocytosed pathogens. Consistent with this idea, others have demonstrated, through interference with exosome-secretion using Rab-27a deficient mice, that exosomes indeed function to promote T-cell immunity during a bacterial infection and are an important source of extracellular antigens[38].

SPPL2a defects trigger anti-CD74 self-reactivity and shape the endosomal architecture

That presentation of self-antigens can lead to autoimmunity is exemplified in **chapter 5**. Here, we show that DCs isolated from patients suffering from the autoimmune condition ankylosing spondylitis stimulate immune responses by presenting self-peptides derived from CD74 at the plasma membrane. CD74, also referred to as invariant chain, is a chaperone protein that is normally only temporarily associated with MHC class II to direct the transfer of newly synthetized MHC class II to antigen loading compartments within the DC, as well as to prevent premature peptide loading. In general, expression of MHC class II is increased under inflammatory conditions. We found that, in ankylosing spondylitis patients, this is accompanied by an increase in surface expression of full length CD74 or the N-terminal fragment CD74-p8 in complex with MHC class II. This is caused by dysfunction of the CD74-processing enzyme signal peptide peptidase-like 2a (SPPL2a) and provokes the immune system to produce autoantibodies to CD74. Of note, SPPL2a deficiency affected endosomal morphology, resulting in constriction of late endosome size upon an inflammatory stimulus. Although we did not analyze EVs

in this chapter, we have previously shown that MHC class II on EVs is not in complex with CD74, but as a heat stable MHC class II/peptide complex[39]. Therefore, it is very well possible that in ankylosing spondylitis patients, MHC class II-p8 complexes are not only present on the plasma membrane, but also on EVs. In this capacity, the p8-presenting EVs could be an important component in the generation and maintenance of the auto-immune response.

Main hypothesis

We hypothesize that MHC molecules present on exosomes released from pathogen-containing compartments are enriched for pathogen-derived peptides, as compared to MHC complexes present on the plasma membrane. This hypothesis is based on our finding that the release of immune-stimulatory EVs by DCs is stimulated though interactions with non-cognate T-cells stimulated (**Chapter 3**) as well as by phagocytosed *E. coli* (**Chapter 4**).

These observations lead us to the following hypothesis describing the physiological role of EVs/exosomes in antigen presentation (**figure 1**): **1.** A DC samples a pathogen in the periphery, and starts to mature. **2.** Upon phagocytosis, phagosomal TLRs are triggered by PAMPS provided by the pathogen. MHC II is recruited to the phagosome, and peptides that are generated by limited proteolysis are loaded into the peptide-binding groove of MHC class II (2.1). Meanwhile, the DC travels towards secondary lymphoid tissues (2.2). **3.** Phagosomal TLR signaling induces Rab27a-mediated and tetraspanin-driven formation of intraluminal vesicles (ILVs) carrying MHC in phagosomes . Consequently, MHC molecules present on phagosomal ILVs are preferentially loaded with pathogen-derived peptides. These ILVs carry costimulatory molecules, ICAM-I, and immune-stimulatory miRNA species. **4.** DCs that have obtained antigens in the skin have been shown to reach the lymph nodes within 6 hours[40]. Based on our kinetic data (**chapter 4**), we suggest that a substantial proportion of pathogen expulsion and associated EV-release will occur in the lymphoid tissue. Fusion of the phagosome with the plasma membrane results in the release of their ILV as antigen presenting exosomes. two different triggers can underly this process: **4.1** Due to sustained intracellular TLR4 triggering, SNAP23-mediated fusion of MVBs with the plasma membrane is induced. **4.2** Within the lymph node, the maturing DCs encounter both activated and non-activated non-cognate T-cells. Encounters with activated non-cognate T-cells result in the release of DC-derived exosomes that carry both antigen presenting molecules as well as immune-stimulatory RNA species. Moreover, these interactions further capacitate DCs for successful activation of cognate T-cells. **5.** In the lymph node, the release of

the antigen presenting exosomes leads towards an increase in and dissemination of the presentation of pathogen-derived peptides, thus enhancing the probability of the activation of cognate T-cells. Upon their release, the exosomes can have different fates: **5.1.** a substantial part of the exosomes may remain attached to the plasma membrane[41], resulting in a local enrichment pathogen-derived peptides in complex with MHC class I and II molecules . Alternatively, exosomes may also disseminate pathogen-derived peptides within the lymph node: **5.2.**, These may bind bystander DCs, where they remain bound to the plasma membrane and thus present antigens **5.3** Alternatively, exosomes can bind CD4+ T-cells, involving the interaction between ICAM-I-LFA-1 interactions[6] **5.4** Lastly, exosomes can be acquired by follicular DCs, enabling presentation of pathogen-derived peptides to B-cells, resulting in an antibody response. While being present at the plasma membrane of (follicular) DCs and T-cells, the exosomes can activate cognate T-cells either directly or with the help of activated bystander DCs. The dense packing of MHC molecule presenting relevant (pathogen-derived) peptides on the exosomes facilitates their efficacy. Conversely, MHC molecules expressed on the plasma membrane of DCs are predominantly loaded with self-peptides. Therefore, these cells are less likely to interact with T-cells recognizing pathogen-derived peptides.

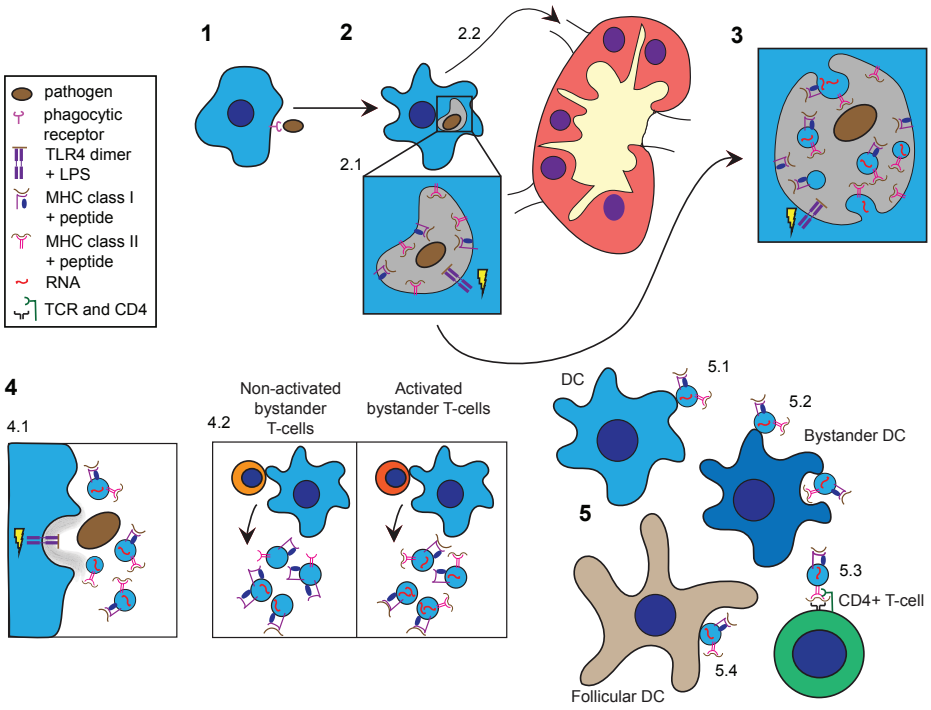


Figure 1: Antigen presentation by DC-derived EVs.
1. An immature DC is activated by the encounter with a pathogen and starts to mature. **2**. The DC phagocytoses the pathogen and phagosomal TLRs are triggered by PAMPs provided by the pathogen. Meanwhile, the DC travels towards secondary lymphoid tissues. **3**. TLR-signaling triggers the generation of ILV in phagosomes in close vicinity of the phagocytosed pathogen, resulting in preferential loading of pathogen derived peptides onto ILV associated MHC molecules. These ILV also carry co-stimulatory molecules, integrins, as well as immune-stimulating miRNA species. **4**. Once in the lymphoid tissue, the phagosome fuses with the plasma membrane due to sustained TLR triggering, releasing the pathogen as well as its ILV, now referred to as exosomes. Alternatively, the release of exosomes can be triggered by activated T-cells. **5**. Upon release, exosomes may remain attached to their parental DC, be recruited by bystander (follicular) DCs or activated T cells. Antigen specific T cells can subsequently become activated by exosomes carrying cognate peptide-MHC complexes. This can occur either directly, or with the help of associated DCs.

Future Perspectives

In conclusion, we identified triggers that induce the release of immune-stimulatory EVs by moDCs. Furthermore, we endeavored to define some of the characteristics of immune-stimulatory EVs. We found that non-cognate interactions between activated T-cells and DCs results in the release of EVs with potent immune-stimulatory functions

as well as the capacity to present antigens. Furthermore, we describe that the release of EVs equipped with antigen-presenting molecules is induced by phagocytosis and expulsion of (inactivated) pathogens. Both findings support a pivotal role for DC-derived EVs in the induction of cellular immunity/adaptive immune responses in the lymph nodes, while preventing auto-immunity. These experimental data culminate in the hypothesis on the role of antigen-presenting EVs in the immune-system as presented above. However, there are many challenges to fully submit this hypothesis to experimentation. In order to be able to translate our results to the human immune system, we resolved to exclusively work with primary, human cells. This resolution brought about several challenges, which we expect will be overcome with current and future technological developments.

Firstly, due to the large number of cells needed to obtain large enough quantities of EVs, we could not work with mDCs or pDCs, but found ourselves limited to the use of monocyte-derived DCs, which are (probably) most related to inflammatory DCs[42,43]. Furthermore, the choice for primary human cells also introduced donor variability. This variability is most apparent from quantitative immunoblot data. However, while assessing the capacity of DC-derived EVs to activate cognate T-cells (**chapter 3**), all donors showed strikingly similar qualitative responses. Moreover, viability is an important factor in EV-release: reduced viability/apoptosis is accompanied by the release of apoptotic bodies, which are considered a subtype of microvesicles[44,45]. We limited the impact of this heterogeneity in our experiments by only including batches of moDCs that displayed >90% viability.

Importantly, the choice for primary human cells obliged us to forego the possibility to genetically manipulate the cells. As a result, although our experimental work sheds light on triggers for EV release, many questions on mechanistic and functionality aspects remain. For example, we cannot exclude that we have foregone analysis of a large portion of EVs that we failed to analyze, as many may have been taken up by target cells, or remained attached their parental cell upon release. Indeed, association with target cells (DCs and T-cells) is mediated via ICAM-1/LFA-1 expression, and interference with this interaction also negatively affects the immune-modulatory functionality of EVs[6,46,47]. Furthermore, EVs released by HeLa cells were shown to remain bound to their parental cell on a large scale, involving a molecule named tetherin[41]. Of note, this capacity may contribute to their activity, as exemplified by a study wherein EVs derived from tetherin k.o. DCs were shown to be compromised in their ability to stimulate CD4+ T-cells[48].

The heterogeneity of EVs further limits a comprehensive analysis[23,49]. It would be advantageous to be able to specifically silence the production of specific EV-subsets (exosomes versus MV) for a better understanding of their mechanistic contribution in immune regulation. Out of the myriad of molecules involved in the biogenesis and trafficking of EVs, the GTPase Rab27a might be the most interesting. Rab27a was shown to specifically drive exosome-release[50], mediate trafficking of pathogen-derived material into vesicles[38], and melanoma cells that expressed low levels of Rab27a released EVs that were less pro-invasive[51].

The main fundament of our hypothesis is the premise that DC-derived EVs, which derive a from pathogen-containing endosomal compartment, are enriched for MHC molecules presenting pathogen derived peptides, as compared to the MHC molecules present on the plasma membrane. In theory, this could be tested by comparing the peptidome presented on the plasma membrane, with the peptidome present on EVs, using mass spectrometry analysis (MS)[52,53]. However, such analysis requests the input of a large number of MHC molecules, a condition that is yet difficult to meet. Firstly, we find ourselves limited by the quantity of EVs that can be isolated from the culture supernatant. Furthermore the efficiency of immunoprecipitation of MHC class I and II, as well as peptide elution from these isolated molecules further diminish the amount of peptide available for MS analysis. We anticipate that further optimization of EV-isolation, as well as both the sensitivity of MS techniques and the isolation of peptide-MHC complexes from EVs may enable such pivotal experiments in the future.

Isolated DC-derived EVs have been clinically tested as vaccines. For example, infusion of DC-derived EVs has been shown to provide protection against infectious diseases[54,55], as well as induction of an immune response to cancer[56–58]. However, instead of infusing *ex vivo*-generated EVs, their *in vivo* induction as part of the vaccination strategy might prove to be more effective.

Vaccines are used to induce an adaptive immune response, protecting humankind as well as livestock against potentially dangerous pathogens. Live, attenuated pathogens can be used for vaccination, as they induce strong cellular immunity without, in most cases, causing disease-like symptoms[59]. However, for safety reasons, this strategy is not feasible for all pathogens. In those cases, vaccines consisting of (parts of) inactivated pathogens can be formulated, but these are poorly immunogenic in absence of adjuvants[60] . Since the 90's, many different adjuvants with different immune-stimulatory mechanisms have been developed[61]. However, apart from the immune-stimulatory characteristics of the adjuvants, their localization relative to the antigen may also be key

to the efficacy of the vaccine. Indeed, a recent study revealed that antigens that were associated with an adjuvant induce a stronger humoral and cellular immune responses[62]. Based on our results, we hypothesize that phagocytosis as well as intracellular TLR-signaling are two key triggers for the induction of antigen-presenting EV-release. We propose that the induction of antigen-presenting EVs plays an important role in the induction of the adaptive immune response Therefore, vaccine-efficiency could be improved by providing pathogen-derived particles that induce phagocytosis and carry TLR stimuli, triggering TLR-activation in phagosomes, and the release of immune potent exosomes.

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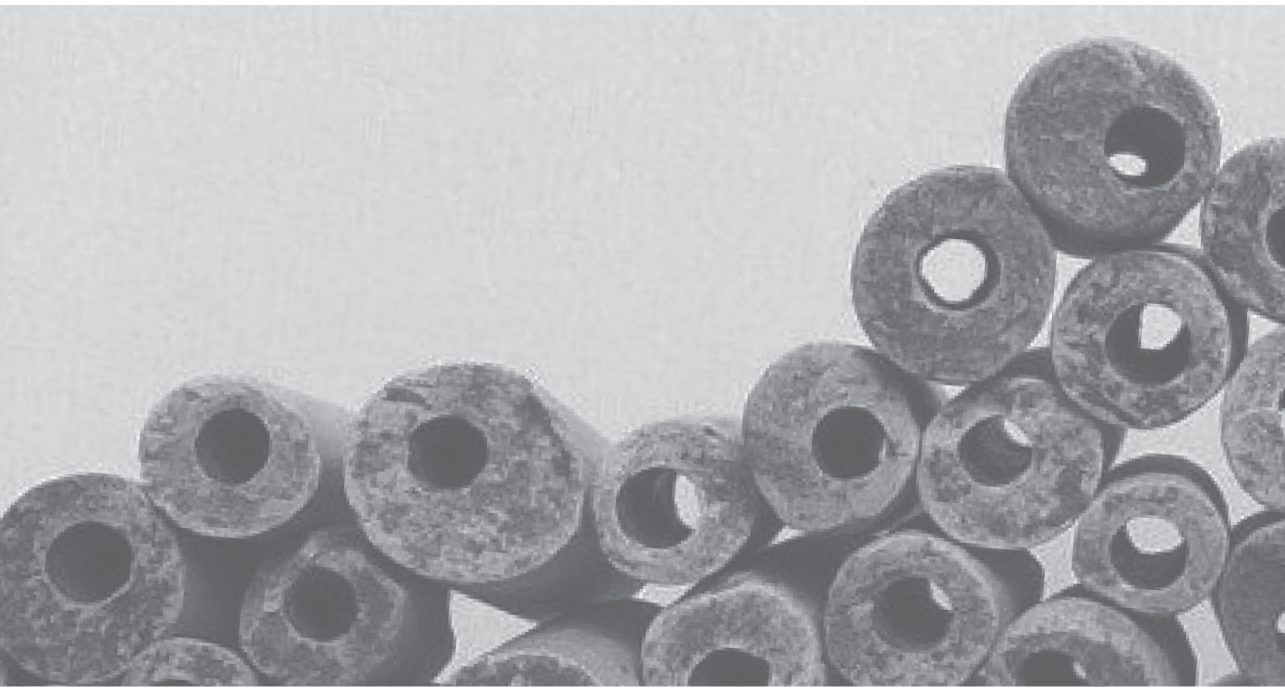


A

Addendum

Nederlandse samenvatting voor niet-ingewijden

Stimulatie van de productie van extracellulaire membraanblaasjes door dendritische cellen: implicaties voor antigeenpresentatie

Het immuunsysteem beschermt mens en dier tegen bedreigingen die zowel van buiten als van binnen het lichaam kunnen komen. Van buiten trachten bacteriën, schimmels en virussen, ook wel pathogenen genoemd, het lichaam binnen te dringen. Bedreigingen vanuit ons eigen lichaam worden gevormd door de ontsporing van de regulatie van celdeling waardoor kanker kan ontstaan. Het immuunsysteem is er op gericht om feilloos onderscheid te maken tussen aan de ene kant bekende, lichaamseigen moleculen, en aan de andere kant onbekende, lichaamsvreemde moleculen. Het immuunsysteem bestaat uit twee pijlers; de aspecifieke (innate) en de specifieke (adaptieve) afweer. De aspecifieke afweer herkent sommige, meer algemeen voorkomende, lichaamsvreemde moleculen en vormt een eerste barrière tegen pathogenen. Na het signaleren van dergelijke moleculen, alarmeert de aspecifieke afweer de specifieke afweer. De celtypes die onderdeel uit maken van de specifieke afweer zijn gespecialiseerd in het snel opruimen van specifieke pathogenen en ontspoorde lichaamseigen cellen. Communicatie tussen deze twee pijlers van het immuunsysteem is daarom essentieel en wordt, onder andere, mogelijk gemaakt door dendritische cellen.

T-cellen

Het adaptief immuunsysteem bestaat uit meerdere celtypes. Een belangrijk celtype is de T-cel. Dit celtype kan onder andere zieke cellen herkennen en opruimen. T-cellen zijn afkomstig uit het beenmerg en rijpen in de thymus. T-cellen kunnen met behulp van receptoren op het celoppervlak specifieke moleculen herkennen, zogenaamde peptiden. In aanleg herkent elke T-cel een ander peptide. Voordat T-cellen vanuit de thymus het bloed in gaan, wordt er geselecteerd op T-cellen die geen lichaamseigen peptiden herkennen. T-cellen die wel lichaamseigen peptiden herkennen worden uitgeschakeld om auto-immuniteit te voorkomen. In grote lijnen zijn er twee soorten T-cellen: zogenaamde helper T-cellen, en cytotoxische T-cellen. Helper T-cellen hebben onder andere als functie geactiveerde B-cellen te stimuleren om te delen en antigen-specifieke antilichamen te produceren. Cytotoxische T-cellen kunnen andere cellen, die een lichaamsvreemd peptide presenteren doden. Zodra een T-cel een peptide herkent, raakt de T-cel geactiveerd. Dit betekent dat de T-cel zich gaat delen en differentiëren, en zo een uitgebreide adaptieve immuunrespons mogelijk maakt. Zoals eerder gezegd

vormen dendritische cellen een link tussen de twee pijlers van het immuunsysteem. Dit is omdat dendritische cellen nodig zijn voor de activatie van T-cellen.

Dendritische cellen

Dendritische cellen bevinden zich overal in ons lichaam en verzamelen voortdurend informatie uit hun omgeving. Dit doen ze door hapjes extracellulair materiaal op te nemen. Dit kunnen moleculen, hele pathogenen of stukken van lichaamseigen cellen zijn. De opgenomen eiwitten worden in de dendritische cel tot peptiden geknipt. Vervolgens worden deze peptiden met behulp van "Major Histocompatibily Complex" (MHC) moleculen, aan de buitenkant van de dendritische cel tentoongesteld. Op die manier maken ze het andere cellen van het adaptieve immuunsysteem, waaronder T-cellen, mogelijk om eventueel lichaamsvreemde peptiden, ook wel antigenen genoemd, te herkennen en daarop te acteren.

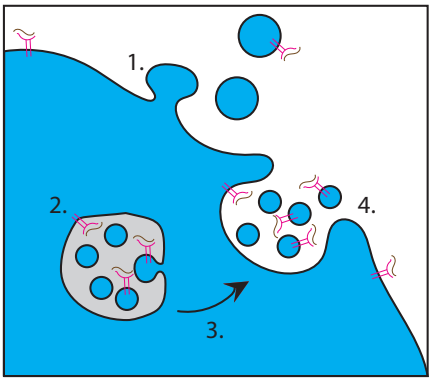
Er zijn grofweg twee soorten MHC moleculen: MHC klasse I en MHC klasse II. De eerste soort, MHC klasse I, is aanwezig op alle cellen in ons lichaam. Deze moleculen presenteren peptiden die van binnen uit de cel verkregen zijn. Dendritische cellen vormen hierin een uitzondering omdat deze ook peptiden die zij vrijmaken uit materiaal dat ze uit hun omgeving hebben opgenomen kunnen presenteren met behulp van MHC klasse I, dat heet cross-presentatie. MHC klasse II komt niet op alle cellen voor, maar specifiek op enkele celtypes die onderdeel uitmaken van het immuunsysteem, waaronder dendritische cellen. Op MHC klasse II moleculen worden peptiden gepresenteerd die afkomstig zijn van eiwitten die van buiten de cel opgenomen zijn. Het is belangrijk om te beseffen dat het overgrote deel van de peptiden die op de MHC moleculen gepresenteerd worden niet lichaamsvreemd zijn. Enkel wanneer er sprake is van een infectie, of mutaties (in het geval van kanker), worden er peptiden gepresenteerd die lichaamsvreemd zijn.

Afhankelijk van het materiaal dat opgenomen wordt, kunnen de dendritische cellen geactiveerd raken. Dit kan omdat dendritische cellen veel verschillende typen receptoren zowel aan de binnen als de buitenkant van de cel dragen die geconserveerde moleculaire patronen van pathogenen of zieke cellen herkennen. Zodra de dendritische cel een dergelijk patroon detecteert, raakt deze geactiveerd. Dit houdt onder andere in dat in korte tijd nog meer materiaal uit de omgeving opgenomen wordt, om als peptide aan de buitenkant van de cel, middels MHC moleculen, gepresenteerd te worden. Na stimulatie migreert de dendritische cel naar de lymfeknopen of de milt waar zich grote aantallen rustende T-cellen bevinden. Geschat wordt dat elk mens wel een miljard verschillende soorten rustende T-cellen heeft, ieder met een eigen specificiteit om

een specifiek MHC/antigeen complex op een dendritische cel te herkennen. Wanneer een MHC gebonden antigeen op een dendritische cel wordt herkend door een specifieke rustende T-cel, wordt deze T-cel geactiveerd, en daarmee het adaptieve immuunsysteem.

Membraanblaasjes

Alle cellen in ons lichaam kunnen kleine membraanblaasjes loslaten. Deze blaasjes kunnen binnen de cel, in zogenaamde endosomen, gemaakt zijn, en daarna worden uitgescheiden via secretie (exosomen), of direct worden afgesplitst van de plasma membraan (microvesicles) (zie figuur 1). Beide typen membraanblaasjes zijn gevuld met cytosol en dragen een grote variëteit aan moleculen bij zich. De compositie is afhankelijk van verschillende factoren: uit welke type cel ze komen, de activatiestatus van die cel, en of het exosomen of microvesicles betreft. Bijna 20 jaar geleden is ontdekt dat membraanblaasjes die door dendritische cellen losgelaten worden peptiden kunnen presenteren op MHC moleculen, en kunnen bijdragen aan de activatie van T-cellen. Het is op dit moment echter nog niet bekend hoe de productie deze blaasjes wordt gereguleerd. Er is ook nog geen duidelijk antwoord op de vraag of en hoe verschillende stimuli, en zo ja welke, het adaptieve immuunsysteem kunnen beïnvloeden. In dit proefschrift staat beschreven hoe wij getracht hebben antwoord te krijgen op zulke vragen.



Figuur 1: Vorming van microvesicles (MV) en extracellulaire vesicles (EV).
1. Microvesicles (MV) ontstaan door directe afsplitsing van het plasmamembraan. 2. Exosomen worden gevormd door binnenwaartse afsplitsing van het membraan van endosomen. 3. Deze endosomen kunnen fuseren met het plasmamembraan. 4. De endosomale membraanblaasjes worden zo losgelaten uit de cel en heten nu exosomen. MHC -peptide complexen zijn niet alleen op het oppervlak van de cel aanwezig, maar ook op microvesicles en (met name) exosomen. Deze kunnen dus ook bijdragen aan antigeen-presentatie.

Vraagstelling

In de meeste gevallen biedt onze afweer voldoende bescherming zonder dat er auto-immuniteit ontstaat. Echter, dit ligt niet voor de hand. Zoals hierboven al gesteld presenteert de overgrote meerderheid van de MHC moleculen lichaamseigen peptiden. In het geval dat een dendritische cel een pathogeen tegen komt en geactiveerd raakt, blijft de dendritische cel ook lichaamseigen peptiden presenteren, zodat deze cel nu in principe ook antigeen-specifieke T-cellen zou kunnen activeren. Daarmee ontstaat een potentieel gevaarlijke situatie waarin auto-immuunreacties geïnduceerd kunnen worden. Dit blijkt echter (meestal) niet te gebeuren en in het afweersysteem bestaan er verschillende mechanismen die voorkomen dat auto-immuunreacties plaatsvinden en tot ziekte leiden. Er zijn door de jaren heen meerdere van deze mechanismen ontrafeld, maar de mechanismen van zogeheten immuuntolerantie zijn nog niet volledig opgehelderd. Dit proefschrift omvat het onderzoek dat ik de afgelopen jaren op dit gebied gedaan heb. Zo hebben we onderzocht of de presentatie van antigenen door van dendritische cellen afkomstige extracellulaire membraanblaasjes bijdragen aan het proces van antigeen-specifieke afweer, van belang in situaties van immuniteit en auto-immuniteit.

Hoofdstuk 2 bestaat uit een uitgebreide beschrijving van de beschikbare literatuur over antigeenpresentatie door membraanblaasjes die afkomstig zijn van verschillende celtypes in ons immuunsysteem. Dit literatuuronderzoek vormt een basis voor hypotheses die getest zijn in de experimentele hoofdstukken.

In de lymfeknopen interacteren dendritische cellen veelvuldig met T-cellen. In **hoofdstuk 3** hebben wij de hypothese getest of zulke interacties tussen dendritische cellen en T-cellen de productie van membraanblaasjes door dendritische cellen stimuleert. Daartoe hebben we zogenaamde monocyten uit bloed van gezonde donoren geïsoleerd, en deze cellen laten uitgroeien tot dendritische cellen. Uit bloed van dezelfde donoren hebben we ook de T-cellen geïsoleerd. We hebben waargenomen dat de productie van membraanblaasjes werd beïnvloed wanneer de dendritische cellen werden geactiveerd door LPS, een bacterie specifiek molecuul. Echter, geactiveerde dendritische cellen gingen nog meer membraanblaasjes uitscheiden in de aanwezigheid van geactiveerde T-cellen. Dit effect was onafhankelijk van antigeen presentatie. We hebben deze membraanblaasjes geïsoleerd uit het kweekmedium, en hun moleculaire compositie bestudeerd. Naast MHC moleculen bevatte deze ook andere eiwitten die karakteristiek zijn voor de productie van extracellulaire blaasjes of de binding daarvan aan T-cellen. Daarnaast bevatten deze blaasjes microRNA moleculen met immuun-stimulatoire eigenschappen. Verder vonden wij dat wanneer MHC klasse I op deze

membraanblaasjes werd beladen met een virus specifiek peptide, deze blaasjes, net als de dendritische cellen waarvan zij afkomstig waren, peptide specifieke T-cellen konden activeren. Deze waarneming bevestigt dat de release van functionele van antigen presenterende membraanblaasjes door dendritische wordt gestimuleerd in een omgeving waar veel geactiveerde T-cellen aanwezig zijn, een conditie die je in een lymfeknoop kunt verwachten.

Wanneer dendritische cellen micro-organismen detecteren kunnen zij deze opnemen via fagocytose; zij komen dan terecht in een fagosoom alwaar zij kunnen worden gedood. In het fagosoom worden met behulp van enzymen peptiden vrijgemaakt die kunnen worden beladen op MHC klasse II. In **hoofdstuk 4** laten wij met twee verschillende methoden zien dat dendritische cellen ook in staat zijn om gefagocyteerde bacteriën (*E. coli*) weer uit te scheiden. Uit onze kwantitatieve experimenten blijkt dat 40% van de opgenomen bacteriën binnen 13 uur werd losgelaten. Daarnaast vonden we dat er in die periode ook sprake was van een toename in de hoeveelheid uitgescheiden membraanblaasjes. Vanwege de coïncidentie van deze twee processen, en de colokalisatie van exosoom-markers in *E.coli* bevattende fagosomen vermoeden wij dat de uitgescheiden membraanblaasjes exosomen zijn die werden gevormd in fagosomen. Wanneer exosomen worden gevormd in bacterie bevattende fagosomen wordt de kans vergroot dat de MHC moleculen op deze exosomen worden beladen met peptiden die van de bacterie afkomstig zijn. Dit laatste hebben we nog niet kunnen bewijzen maar zou een zeer doelmatig mechanisme zijn om de efficiëntie en selectiviteit van antigeen presentatie te vergroten. In de toekomst hopen we bovenstaande hypothese te testen door middel van massaspectrometrie, een techniek waarmee de op MHC moleculen gepresenteerde peptiden kunnen worden geïdentificeerd.

Zoals eerder genoemd werkt het immuunsysteem niet in alle gevallen feilloos om onderscheid tussen lichaamsvreemde en lichaamseigen peptiden te maken. In **hoofdstuk 5** onderzoeken we een mechanisme dat ten grondslag ligt aan een auto-immuunreactie in patiënten met ankyloserende spondylitis (AS). Deze ziekte is geassocieerd met herkenning van het lichaamseigen “Class II MHC-associated invariant chain peptide” (CLIP). CLIP is een peptide fragment van het chaperone eiwit “invariant chain”, welke pas gesynthetiseerde MHC klasse II moleculen begeleidt naar endosomen, waar de MHC klasse II moleculen beladen worden met antigeenpeptide. Het bijzondere aan invariant chain is dat het CLIP fragment, zij het met relatief lage affiniteit, zelf de plek in kan nemen van de peptide-bindende locatie in MHC klasse II moleculen. Nadat het zijn rol heeft gespeeld in het begeleiden van intracellulair transport van MHC klasse II moleculen naar endosomen wordt invariant chain binnenin een deel

van deze celorganellen, de “late endosomen”, in opeenvolgende stappen van precieze afbraak steeds kleiner geknipt. Uiteindelijk blijft op die manier, in de gezonde situatie, alleen het CLIP peptide over in de peptide-bindende locatie van het MHC klasse II molecuul. Tenslotte wordt het CLIP op MHC klasse II moleculen vervangen door antigeen-peptiden. Dit knippen van eiwit wordt uitgevoerd door verschillende enzymen in de endosomen, waaronder signal peptide peptidase-like 2A (SPPL2a). Wij hebben gevonden dat SPPL2a niet goed functioneert in AS-patiënten: Zodra door activatie van de cel de synthese van nieuwe MHC klasse II moleculen toeneemt, accumuleren MHC klasse II moleculen met niet volledig geknipte fragmenten van invariant chain op het plasma membraan. In AS-patiënten is er sprake van ontstekingen, en dit leidt tot meer cel-activatie en toename van de synthese van nieuwe MHC klasse II moleculen. Ten gevolge daarvan worden monocyten in AS-patienten versterkt geactiveerd. Dit heeft in het geval van verminderde SPPL2a werking tot effect dat er meer MHC klasse II moleculen gebonden door invariant chain peptidefragmenten op het plasmamembraan van monocyten aanwezig zijn. We vonden dat als gevolg hiervan, 3 op de 4 geteste AS patiënten antilichamen tegen CLIP in hun bloed hadden. Wij denken dat dit als volgt te verklaren is: in de normale situatie zijn er weinig MHC klasse II complexen met CLIP aanwezig op het plasmamembraan. In het geval dat SPPL2a verminderd werkt, neemt dit aantal toe, met een grotere kans op in herkenning door CLIP specifieke T-cellen, resulterend in autoimmuniteit.

De hierboven beschreven bevindingen worden samengevat en in de context van bestaande literatuur geplaatst in **hoofdstuk 6**. Verder doen wij in dit hoofdstuk ook suggesties voor toekomstig onderzoek.

Conclusie en toekomstperspectief

We zijn aan dit project begonnen om de volgende vraag te beantwoorden: hoe kan het dat ons adaptief immuunsysteem, in de vorm van antigeen-specifieke T-cellen, zo feilloos de weg kan vinden naar een relatief klein aantal lichaamsvreemde peptiden in een zee van lichaamseigen peptiden? Hiertoe hebben we onderzocht of (een deel van) het antwoord ligt in de presentatie van peptiden door van dendritische cellen afkomstige membraanblaasjes. We hebben gevonden dat zowel interacties met geactiveerde T-cellen, als opname en weer uitspugen van bacteriën de release van membraanblaasjes en de daarmee geassocieerde moleculen, waaronder antigeen-presenterende moleculen, stimuleert. Daarnaast weten we nu dat de membraanblaasjes die door geactiveerde T-cellen geïnduceerd worden efficiënter zijn in het induceren van een immuunrespons dan wanneer ze geïnduceerd zijn door niet geactiveerde T-cellen. Verder hebben we aanwijzingen dat er exosomen worden gevormd in bacterie

bevattende fagosomen. Dit impliceert dat de MHC moleculen op deze blaasjes wellicht verrijkt zijn met van de bacterie afkomstige antigenen.

Onze bevindingen kunnen, na verdergaand onderzoek, mogelijk resulteren in medische toepassingen, bijvoorbeeld voor het ontwerpen van nieuwe effectieve vaccins. Door onze experimenten kennen we nu enkele voorwaarden voor het genereren van antigeen-presenterende membraanblaasjes. Daarnaast hebben wij indicaties dat deze blaasjes een belangrijke rol zouden kunnen spelen in preferentiële presentatie van antigenen die in pathogeen bevattende fagosomen worden gegenereerd. Dit zou kunnen betekenen dat nieuwe vaccins, die bestaan uit partikels waaraan zowel antigenen als een adjuvans dat een endosomaal gelokaliseerde receptor kan activeren zijn gekoppeld, een betere effectiviteit hebben dan bestaande vaccins.

About the author

Marthe Frasquita Sophie Lindenbergh was born on April 20th 1990 in Groningen. She grew up in Wehe – den Hoorn, a small village in the north of Groningen. After finishing secondary school (Praedinius Gymnasium, Groningen), Marthe moved to Utrecht in 2008 to start the bachelor Biomedical Sciences (BSc 2012), and continued her studies with the master Infection and Immunity in 2012 (MSc 2014). During her Master studies, Marthe participated in the honours program organized for the Life Sciences Masters. As part of this program she visited the lab of J.P. Roose at UCSF (University of California, San Francisco) for a research internship of 8 months. In the beginning of 2014 Marthe wrote a proposal for a PhD project to take place in the labs of Dr. M.L. Boes at the University Medical Center Utrecht and Prof. Dr. W. Stoorvogel at the faculty of Veterinary sciences, Utrecht University. This proposal was awarded with an NWO Graduate Program Grant by the Infection and Immunity graduate school, and Marthe started working on the project in October 2014.

During the next four years, Marthe investigated the mechanisms underlying the induction of the release of DC-derived extracellular vesicles, as well as the activity of these EVs in initiating immune reactions. During this period, she strived to work as autonomously as possible, thus learning from mistakes in order to become an independent scientist .

After finishing her PhD-contract, Marthe started working as a trainee patent attorney at V.O. in Utrecht. Here she trains to advise and aid inventors in the life sciences in protecting intellectual property, by carefully turning unique findings and ideas into patents and strategies.

Aside from her professional career, Marthe likes to challenge herself in several hobbies. She started playing violin at the age of 5, and later switched to viola. She still very much enjoys to play in orchestras. A bigger hobby is sports; during her studies Marthe rowed competitively for 2 years and coached competitive rowers for 3 years at U.S.R “Triton”. Since several years Marthe competes in (cross)triathlons.

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Dankwoord

De afgelopen jaren heb ik met enige regelmaat uitgekeken naar het moment waarop ik het dankwoord voor mijn proefschrift zou mogen schrijven. Het leek me zo'n groots moment, een mooie manier om een belangrijke en ook wel zware periode af te sluiten. Nu het moment daar is, is het net een beetje anders. Het moment is iets minder groots en gewichtig dan gedacht. Toch is het leuk om er op terug te kijken. Het zijn leerzame tropenjaren geweest. Aan het doorkomen van de afgelopen jaren hebben een heleboel mensen aan bijgedragen, en die wil ik hier graag voor danken. Hieronder volgt een lijst bedankjes die, uiteraard, niet uitputtend is!

Willem, wat ontzettend fijn dat we het project samen ten einde hebben gebracht. Het heeft misschien uiteindelijk niet àlles gebracht wat we hoopten, maar hopelijk krijgt het een vervolg in de toekomst. Het is niet altijd makkelijk geweest om samen te werken, niet in de laatste plaats door mijn koppigheid. Ik wil je dan ook bedanken voor al je tijd en geduld.

Marianne, toen ik je net leerde kennen was ik af en toe wel een beetje bang voor je. Inmiddels is dat gelukkig over. Je hebt me geweldig gesteund en gecoacht, niet alleen op wetenschappelijk gebied, maar ook zeker op persoonlijk vlak. De besprekingen (die we, helaas, te weinig hadden) gaven me altijd weer een extra zetje, net wat meer energie, om weer door te buffelen. Heel veel dank voor alle fijne zetjes in de goede richting!

Richard, wat ben jij een fijne stabiele factor geweest. Met alle vragen kon ik bij je terecht, en je had altijd goede ideeën om verder uit te werken. Bovenal heb je onvermoeibaar getoond in het nóg mooier maken van plaatjes en nóg correcter analyseren van data. Ontzettend veel dank hier voor.

Lieve Boes-groep, ook wel "Marianne's weeskinderen": Lotte, Tessa, Sandra, Emmerik, Willemijn, Sarah. Dank voor de Boesgroepmeetings, vol goede suggesties en met best vaak taart. Tessa, wat was het leuk om samen experimenten te doen. Ik wens je heel veel succes met de laatste loodjes van je proefschrift!

Collega's van Biochemie en Celbiologie. Jullie zijn met zoveel dat ik niet iedereen persoonlijk zal noemen, maar ik dank jullie natuurlijk wel allen! Ellen, je was (en ongetwijfeld, bent) een feest om mee samen te werken: supergeorganiseerd én heel erg gezellig. Toch wel jammer dat we nu niet meer samen PBMCs kunnen isoleren. Martijn v. H., een week begon niet goed als we niet even op maandagochtend een diepgaand

gesprek over het leven konden voeren. Jouw wijsheden blijven een bron van inspiratie. Susanne en Tom, bedankt voor het gezamenlijk PhD-student-zijn. Inclusief herkenbare pieken en dalen. Marije, dankzij jou hoefde ik niet vaak alleen te zijn in het (grote) lab. Bedankt voor alle goede gesprekken! Esther V., hartelijk dank voor al het harde werk voor de mooie plaatjes! Martijn M. en Aike, als ik na een lange dag op het lab nog niet kapot was, sleurden jullie me wel mee op de racefiets om dat nog even voor elkaar te boksen. Dank daarvoor. Alberta, dank je wel voor het introduceren van wat Zuid-Europese gezelligheid! En natuurlijk dat je "yes" zei op de vraag of je paranimf wilde zijn.

Het is heel belangrijk gebleken om eventuele stress of bezorgdheden van het werk weg te spoelen met een gezonde dosis hobby's. Om te beginnen muziek. Aanvankelijk mocht ik op maandagavonden frustraties eruit spelen bij het Utrechts Studenten Concert, dank dat jullie dit verdragen hebben. Later kreeg ik de mogelijkheid dit voort te zetten bij het Vignolle ensemble. Jullie zijn van grote therapeutische waarde.

Van nog grotere therapeutische waarde was, en is, sport. Dit begon met de USP hardloopcursus gegeven door triathlon-fenomeen Dirk. Op donderdagavond drilt hij met succes een schare steeds harder lopende hardlopers. Gelukkig was het ook nog gezellig. Door Dirk geinspireerd trok ik ook wat vaker een badpak aan. Zo verscheen badmeester Frans in beeld. Hij was niet te beroerd om donderdag ochtend om 7 uur mij met indrukwekkend volume van tips te voorzien. Uiteindelijk culmineerden deze hobby's in lidmaatschap bij het Utrechtse Heuvelrug Triathlon Team (UHTT). De trainingen op de Utrechtse Heuvelrug zijn nog steeds een garantie voor gezelligheid en pijnlijke spieren.

Voor een grote dosis gezelligheid kon en kan ik altijd bij jullie terecht, Veerle en Jarno! Aanvankelijk als huisgenoot, en later als bevriend stel (wie had dat laatste gedacht!). Met jullie samen kunnen we heerlijk burgerlijk zijn, die principes zijn inmiddels overboord. De poezen hebben we al (met dank aan de charmes van Olly en Daisy). Op een dag zullen wij ook een Airfryer hebben. En jullie een tuinkachel. De etentjes en weekendjes weg samen met jullie waren en zijn een groot feest. Laten we vooral nog lang door modderige rivieren raggen op de mountainbikes, lange avonden tafelen (met veel marshmallows), en veel Mario kart spelen.

Lieve schoonfamilie: Elfriede en Peter, Ernst en Frederike, Iris en Moataz, Julia en Ido. Als laatste werd ik toegevoegd aan jullie grote familie, en ik voel me altijd zeer welkom. Dank voor jullie interesse in mijn bezigheden, en ook de steun als het wat zwaarder was.

Mijn lieve, grote broers: Pieter-Jelte, Ruurd en Sybolt. Gelukkig zijn jullie niet altijd even lief geweest vroeger, zo hebben jullie bijgedragen aan het kweken van een dikke huid. Die is best aardig gelukt. Sommige van jullie wijsheden had ik misschien wat meer ter harte moeten nemen: Pieter-Jelte, je hebt me nog zo gewaarschuwd niet aan een promotietraject te beginnen. Jullie wederhelften: Anne, Maartje, en Neeltje, dank voor alle gezelligheid, interesse, en natuurlijk alle superlieve nichtjes en neefjes!

Lieve Boukje en Johannes. Dank voor de onvoorwaardelijke steun die ik altijd ervaren heb. Nergens is het zo “thuis” als bij jullie. Liters thee, lange avonden bij de kachel met een boek, ’s zomers buiten biertjes met strohoeden. Wandelen over Abelstok om appels te rapen, pijpjes zoeken op het land. De weekenden in het noorden bij de “B&B” met permanente kamer zijn ware minivakanties. Jullie hebben me geleerd om op eigen benen te staan, een brede interesse bijgebracht, het belang van tafelmanieren benadrukt, maar bovenal hebben jullie me geleerd om altijd door te zetten. Dat is meer dan eens van onschatbare waarde gebleken.

Maurits, toen ik aan het promotietraject begon wist ik nog niet beter dan dat je tot het hout-toetervee behoorde. Een half jaar later leerden we elkaar al voetballend en survivallend beter kennen op het dak van de Ikea en in de sloten van de Uithof. Vervolgens vonden we elkaar in de liefde voor Italiaanse ijsjes en Britse autoprogramma’s. Inmiddels heb je het aangedurfd om met me te trouwen. De afgelopen jaren ben je bent soms (gewillig) slachtoffer van mijn frustratiefietstochten geweest. Heb je fietsend mijn therapeutische hardlooptochten vergezeld. Meegewandeld als ik zo nodig in buitenwater wilde zwemmen. Je weet wanneer ik een snickers nodig heb. Je hebt onze twee harige relativeerders (Tido en Loki) in dienst genomen. Je hebt meer dan eens bewezen er onvoorwaardelijk voor me te zijn. Je geeft me zelfvertrouwen wanneer ik het mis. Lieve Maurits, jouw aanwezigheid en niet aflatende steun hebben een hele grote rol gespeeld in het voltooien van dit promotietraject. Je hebt me er als een ware mental coach doorheen geloodsd, en ik weet niet waar ik moet beginnen om je te bedanken. Daarom mag je me vanaf nu tijdens het fietsen altijd vragen om te stoppen voor een stukje appeltaart.