Aus der Klinik für Dermatologie, Venerologie und Allergologie
(Prof. Dr. med. M. P. Schön)
im Zentrum Arbeits-, Sozial-, Umweltmedizin und Dermatologie
der Medizinischen Fakultät der Universität Göttingen

Immunomodulatory activity of murine keratinocyte-derived exosomes

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Kristina Kotzerke
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Dekan: **Prof. Dr. rer. nat. H. K. Kroemer**

1. Berichterstatter: **Prof. Dr. med. M. P. Schön**

2. Berichterstatter/in: **Prof. Dr. rer. nat. H. M. Reichardt**

3. Berichterstatter/in: **Prof. Dr. rer. nat. P. Schu**

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APC	Antigen-presenting cell
APC	Fluorophore: Allophycocyanin
APS	Ammonium persulfate
BMDC	Bone marrow-derived dendritic cell
cDNA	Complementary deoxyribonucleic acid
CFDA-SE	Carboxyfluorescein diacetate succinimidyl ester
CFSE	Carboxyfluorescein succinimidyl ester
DAPI	4′,6-diamidino-2-phenylindole
DC	Dendritic cell
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetate
ELISA	Enzyme-linked Immunosorbent Assay
EM	Electron microscopy
ESCRT	Endosomal sorting complexes required for transport proteins
Exo	Exosome
Exp	Experiment
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte macrophage colony-stimulating factor
HBSS	Hank's balanced salt solution
HEPES	2-(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish peroxidase
IAA	Iodoacetamide
IFNγ	Interferon γ
IL	Interleukin
ILV	Intraluminal vesicle
MACS	Magnetic cell separation
Med	Medium
MDDC	Monocyte-derived dendritic cell
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MPEK	Murine epidermal keratinocyte progenitor cell
MVB	Multivesicular body
NCBI	National Center for Biotechnology Information

OVA	Ovalbumin
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PE	Fluorophore: R-phycoerythrin
PI	Propidium iodide
PRR	Pattern recognition receptor
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-Polyacrylamid gel electrophoresis
TBS-T	Tris-buffered saline-Tween 20
TEMED	Tetramethylethylenediamine
TLR	Toll-like receptor
TNFα	Tumor necrosis factor α
WGA	Wheat germ agglutinin

1 Introduction

1.1 Exosomes

Exosomes are small membrane vesicles of endocytic origin. They have been described for the first time thirty years ago. Early research focusing on reticulocyte maturation and investigation of the destiny of the transferrin receptor discovered accumulation of transferrin receptors in multivesicular bodies and proposed the transferrin receptor to be externalized in 50 nm-vesicles (Harding *et al.* 1983; Pan and Johnstone 1983). These small exocytosed membrane vesicles were termed exosomes (Johnstone *et al.* 1989). For specification of usage, exosomes are defined by three main characteristics: a saucer-like shape and size of 40 - 100 nm as determined by electron microscopy, a density of 1.10 - 1.23 g/ml in a sucrose gradient, and the presence of certain exosomal markers including alix, tsg101, CD81, and flotillin. These markers belong to different protein groups involved in targeting, signaling, or fusion. One of the major protein groups found in exosomes are the so called tetraspanins like CD 81 (Mathivanan *et al.* 2012; Thery *et al.* 2002). A new great interest on exosomes arose about ten years later, when it was discovered that exosomes are involved in immunological processes (Raposo *et al.* 1996). Since this outstanding discovery, the role of exosomes has been addressed in several studies *in vitro* and *in vivo*.

1.1.1 Production, biogenesis, and functions

A variety of cell types produces exosomes: hematopoietic cells such as dendritic cells, mast cells, B and T cells (Thery *et al.* 2002) and also non-hematopoietic cells such as intestinal epithelial cells (van Niel *et al.* 2001), human keratinocytes (Chavez-Muñoz *et al.* 2008), and solid tumor cells (Xiao *et al.* 2012). Exosomes have even been discovered *in vivo* in body fluids such as blood (Caby *et al.* 2005), urine (Zhou *et al.* 2006), and bronchoalveolar lavage (Admyre *et al.* 2003). The biogenesis of exosomes itself has not yet been fully understood and still requires

further clarification regarding its details. Yet, a substantial body of evidence has accumulated that provides insight into the main principles of exosome biogenesis: Eukaryotic cells hold a highly developed endosomal network to link synthesis, transport, and fusion processes. Early endosomes are membrane-bound organelles that either gather endocytosed material from the plasma membrane or synthesized proteins from the Golgi complex. These early endosomes are able to transform to multivesicular bodies (MVB), sometimes also referred to as late endosomes or multivesicular endosomes, through inward budding of their membrane and forming small intraluminal vesicles (ILV). Evidence suggests the existence of different subpopulations of MVB, differing in destinations. MVB can either be directed to fuse with lysosomes for degradation or to fuse with the cells' plasma membrane exocytosing its vesicles. These small exocytosed membrane vesicles are referred to as exosomes (Fig. 1).

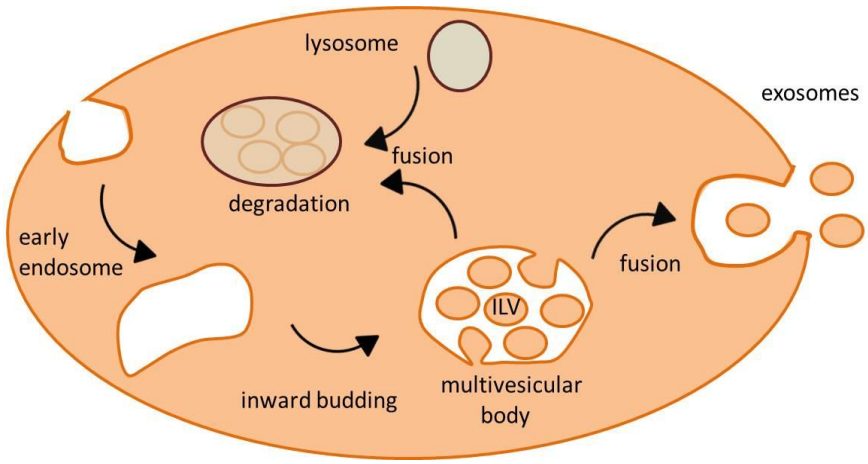

Figure 1: Biogenesis of exosomes. Early endosomes transform to multivesicular bodies by inward budding of their membrane and forming small intraluminal vesicles. Multivesicular bodies either fuse with lysosomes and are degraded or they fuse with the cell membrane and exocytose their vesicles. These exocytosed membrane vesicles are referred to as exosomes.

Due to the mechanism of emergence, exosomes contain material of the cytoplasm and the same membrane orientation as the cell itself, representing a cell in miniature format with regard to its structure and lipid bilayer (Lakkaraju and Rodriguez-

Boulan 2008). However, exosomes can be enriched with certain cargoes in comparison to their mother cells and do not contain the exact same protein composition (Ratajczak *et al.* 2006). A pivotal role in the process of biogenesis has been ascribed to the different endosomal sorting complexes required for transport (ESCRT) proteins. These complexes engineer inward budding of vesicles, cluster marked proteins, and package those proteins into the formed ILV. Alix, often used as classical exosomal marker, holds a linker role between the different ESCRT proteins and might also be involved in the generation of intraluminal vesicles (Babst 2005).

During the years following first discovery, exosomes attracted more and more notice. It became clear that the idea of exosomes functioning as the garbage system of the cell by externalizing obsolete proteins was only one of various functions. As exosomes contain a certain set of exosome-specific proteins, they additionally contain a special set of proteins depending on the cell type they originate from (Simpson *et al.* 2008). Secreted exosomes can be internalized by other cells (Morelli *et al.* 2004) and even a specific uptake and target selection has been suggested (Rana and Zoller 2011). Consequently, exosomes can be considered as a non-classical way of protein secretion and delivery and as a mediator of communication without direct cell-cell contact. By influencing cell-to-cell communication, exosomes are also able to affect and direct immune processes. Further, the enrichment of certain proteins in exosomes qualifies them as usable biomarkers for diseases like Alzheimer`s disease (Cogswell *et al.* 2008), Sjögren`s syndrome (Michael *et al.* 2010) and especially for certain tumor diseases, as exosomes are produced by a variety of tumor cells (Simpson *et al.* 2009). Different studies focusing on dendritic cell-derived exosomes established a revolutionary therapy option using exosomes as vaccine in immunotherapy against cancer (Viaud *et al.* 2010). Lately, different groups were able to demonstrate the transfer of microRNA and messengerRNA in exosomes, even broadening the understanding of exosomes' functions to the possibility of genetic exchange (Valadi *et al.* 2007). Unfavorably, as exosomes deliver information and proteins to different T cells, they can also spread diseases by transporting infectious cargoes such as viruses or prions

(Fevrier *et al.* 2004; Nguyen *et al.* 2003). Besides the above-mentioned functions - communication, immune modulation, biomarker, immunotherapy, transfer of mRNA, transfer of infectious cargo - exosomes might exert additional, yet unknown, functions and further research will be needed for clarification.

1.1.2 Immune functions of exosomes

The strong interest in immunological capacities of exosomes arose in the late nineties with the discovery of B cells presenting antigens via their exosomes (Raposo *et al.* 1996). Subsequent research demonstrated antigen-presenting cell-derived (APC-derived) and tumor-derived exosomes to carry antigenic peptide-loaded MHC I and MHC II molecules and to induce antigen-specific T cell responses *in vitro* and *in vivo* (Chaput and Thery 2011; Thery *et al.* 2009; Zitvogel *et al.* 1998). Even though some exosomes are able to directly interact with T cells to elicit immune responses (van Niel *et al.* 2003), others depend on the presence of APC (Skokos *et al.* 2001). Further, it is believed that immature DC transfer processed antigen-MHC complexes via exosomes to other DC, spreading antigenic information and multiplying the number of effective APC (Pant *et al.* 2012). However, the way how exosomes influence immune reactions strongly depends on the maturation and stimulation stage of the parent cell they are derived of and the cell lineage itself (Viaud *et al.* 2010). These aspects lead either to pro-inflammatory responses as for example demonstrated for B cell-derived and APC-derived exosomes (Admyre *et al.* 2007; Kovar *et al.* 2006) or to anti-inflammatory responses as demonstrated for exosomes found in bronchoalveolar lavage of tolerized mice (Prado *et al.* 2008) or for exosomes derived from serum to treat arthritis (Yang and Robbins 2012). Since most research has been conducted with professional APC it will be interesting to broaden immunological aspects of exosomes by focusing on exosomes derived from non-professional APC as keratinocytes.

1.2 Keratinocytes and the cutaneous immune system

Keratinocytes, constituting the majority of epidermal cells, form a firm united multicellular structure, building up a barrier to environmental influences. Besides the physical barrier to pathogenic microbes, keratinocytes express MHC I and a subset of so called toll-like receptors (TLR), belonging to the group of pattern recognition receptors (PRRs). This kind of receptors is activated by certain components of microbial pathogens named pathogen-associated molecular patterns (PAMPs) (Mempel *et al.* 2007). The activation of TLRs through pathogens induces an intracellular cascade leading to the production of chemokines and cytokines. Those chemokines, in turn, recruit further immune competent cells such as neutrophils to eliminate invading pathogens or at least to prevent their spreading (Iwasaki and Medzhitov 2004). Additionally, keratinocytes form a chemical barrier through the production of antimicrobial lipids. By means of those antimicrobial peptides including defensins, cathelicidins, and RNase7, they are able to kill or inactivate a variety of microorganisms (Pivarcsi *et al.* 2005) (Fig. 2).

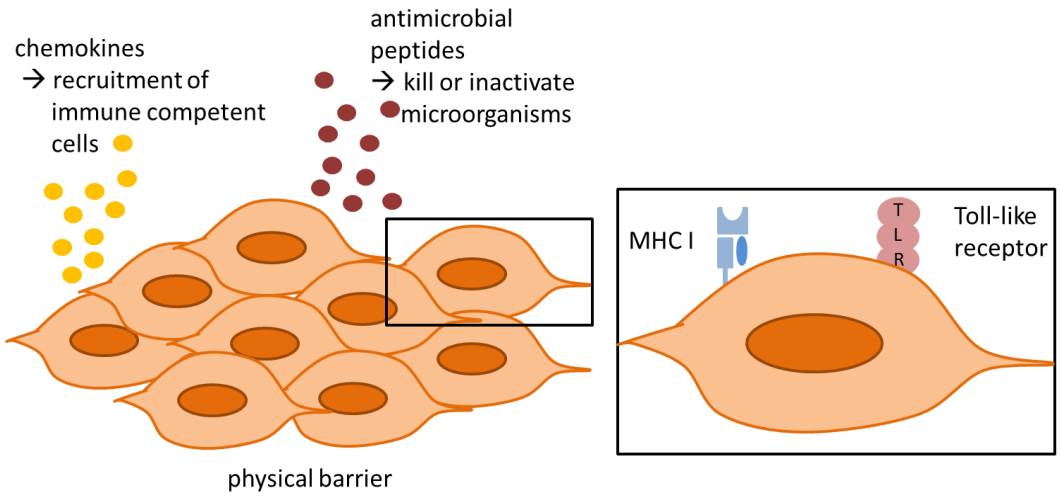

Figure 2: Keratinocytes are an integral component of the cutanous immune system. Keratinocytes express MHC I and a subset of toll-like receptors. Through activation of these receptors, keratinocytes produce different chemokines to recruit immune competent cells. Further, keratinocytes are able to kill or inactivate microorganisms with antimicrobial peptides.

Besides the pivotal role of keratinocytes for cutaneous innate immunity, an important contribution to adaptive immune responses is discussed. Keratinocytes constitutively express MHC I but no MHC II. However, inflammatory conditions (IFNγ) induce keratinocytes to express MHC II and to function as so called non-professional APC, providing co-stimulatory signals to T cells (Nickoloff and Turka 1994). These findings are supported by the discovery that keratinocytes function as accessory APC *in vitro* and *in vivo* irrespective of the presence or absence of professional APC (Kim *et al.* 2009). Moreover, it has been shown that keratinocytes are able to take up antigen, to efficiently process it, and to present it to CD4-positive and CD8-positive memory T cells, thus inducing an immune response (Black *et al.* 2007; Blume *et al.* 2009).

1.3 Adaptive immune response

Keratinocytes accomplish a main task of the cutaneous innate immunity. With the discovery of keratinocytes functioning as non-professional APC, research started to focus on their ability to direct adaptive immune responses. Classically adaptive immune responses are induced by professional APC. In the skin, Langerhans cells fulfill this task (Banchereau and Steinman 1998). Immature dendritic cells have a high phagocytotic activity, thus they efficiently internalize antigen. Uptake of antigen leads to maturation, lowers phagocytotic activity, and increases the expression of co-stimulatory molecules such as CD40 or CD86. The internalized antigen is processed and presented mainly via MHC II to CD4-positive T cells but also by cross-presentation via MHC I to CD8-positive T cells, inducing an antigen-specific T cell response (Mellman and Steinman 2001) (Fig. 3).

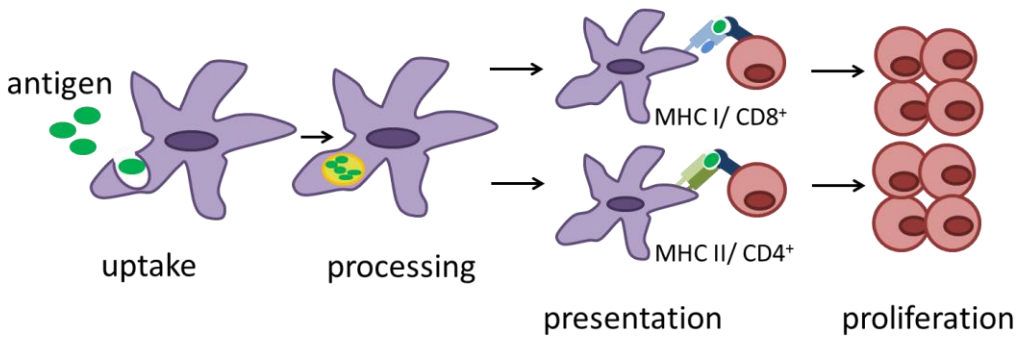

Figure 3: Overview of an adaptive immune response. APC take up antigen, process it, and present it via MHC II to CD4-positive T cells or as cross-presentation via MHC I to CD8-positive T cells, inducing and antigen-specific T cell response.

2 Aim of this study

Research into the (patho)-physiology of exosomes has focused primarily on exosomes derived from hematopoetic cells (Thery *et al.* 2002). Hence, data on exosomes of epithelial cells such as keratinocytes are scant (Chavez-Muñoz *et al.* 2008; Chavez-Muñoz *et al.* 2009; Sanderson *et al.* 2008). The aim of this study was to explore keratinocyte-derived exosomes as a new mechanism for keratinocytes to influence immunity. As keratinocytes are organized in a firm polarized epithelial tissue structure, they only have a close range to present antigen to other cells when acting as non-professional APC. To expand their range of communication with other cells, keratinocyte-derived exosomes could serve as an additional mechanism to spread antigenic information to certain cells beyond their immediate reach. Experimental evidence suggests that the uptake of exosomes is a non-random event influenced by the presence of certain tetraspanins as uptake selection markers (Rana and Zoller 2011). Consequentely, keratinocytes could take up antigen and transfer it to their exosomes. These antigen-loaded exosomes could be taken up by dendritic cells and influence their cytokine profile or maturation state. Further, antigen-containing exosomes internalized by dendritic cells could be presented to antigen-specific T cells and elicit antigen-specific proliferation (Fig. 4).

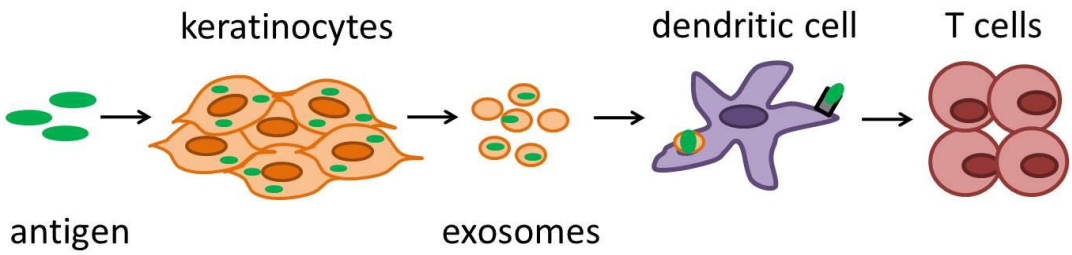

Figure 4: Hypothetic role of keratinocyte-derived exosomes in immune responses. Keratinocytes take up antigen and transfer it to their exosomes. These antigen-containing exosomes then become internalized by dendritic cells and elicit an antigen-specific T cell response when presented.

In reverse, to address the mutual influence of exosomes and their environment, the influence of environmental conditions on exosome composition should also be explored. As frequent chronic diseases, the pathologically altered cytokine environments in psoriasis and atopic dermatitis appear suitable for this investigation (Bieber 2012; Raychaudhuri 2012). Toward this end, the follwing questions should be answered to address the immunlogical role of keratinocyte-derived exosomes:

- Do keratinocytes transfer internalized antigen to their exosomes?
- Do these exosomes influence the maturation state and interleukin profile of bone marrow-derived dendritic cells (BMDC)?
- Are antigen-containing exosomes able to elicit an antigen-specific T cell response via BMDC?
- Do environmental conditions influence the composition of keratinocyte derived exosomes?

3 Material and Methods

3.1 Material

3.1.1 Laboratory equipment

Table 1: Laboratory equipment

Laboratory equipment	Company
Appliskan Multimode Microplate Reader	Thermo Scientific
Autoclave	Sanyo
BD FACSCanto II	BD Bioscience
Beckman optima LE 30K ultracentrifuge	Beckman Coulter GmbH
bucket rotor Sw 32Ti	Beckman Coulter GmbH
Centrifuge Multifuge 15-R	Heraeus
Centrifuge Pico 17	Heraeus
Centrifuge Rotina 35	Hettich Zentrifugen
CM120 electron microscope	Philips
CO_2 incubator HERAcell 150i	Thermo Scientific
Confocal microscope Olympus Fluoview 1000,	Olympus
Copper grid	Plano
Cytospin 2 centrifuge	Shandon
Double distilled water system arium® 611VF	Sartorius
Electrophoresis Power Supply-EPS 1001	Amersham Biosciences
Heating block Thermo Stat plus	Eppendorf
Incubator HERAcell 150	Thermo Scientific
Incubator HERAcell 150i	Thermo Scientific
iTEM-Camera	Olympus
LAS-4000 imaging system	Fujifilm
LTY Orbitrab XL hybrid mass spectrometer	Thermo Scientific
MACS MultiStand	Miltenyi
MidiMACS Separator	Miltenyi
Magnet stirrer	VWR
Micro scale ALC	Acculab
Microcentrifuge	Sprout
Microscope Axio Imager M1	Zeiss
Microscope Axiovert 40	Zeiss
Nanoflow LC system	Agilent
Neubauer counting chamber	Brand
pH-meter FE20-Five easy™	Mettler Toledo
Photometer Genesys 10 Bio	Thermo Scientific
Pipetboy acu	IBS Integra Biosciences

Pipette set: 1000, 100, and 10 µl	Eppendorf
PowerPac Basic	Bio-Rad
Refrigerated centrifuge	Eppendorf
Refrigerator and freezer +4°C, -20°C, -80°C	Liebherr
Roborack-96	Micronic
Roborack-96 cover	Micronic
Rocking platform Polymax 1040	Heidolph
Scale precision	Sartorius
Scale Vicon	Acculab
Shaker	Grant-bio
See-saw rocker SSL4	Stuart
Sonorex RK 100 Transistor	Bandelin
Spectrometer Genesys 10Bio	Thermo Scientific
Sterile bench HERAsafe	Thermo Scientific
Sterile bench Safe 2020	Thermo Scientific
Thermal mixer ThermoStat plus	Eppendorf
Trans –Blot SD semi-dry transfer cell	Bio-Rad
Vacusafe comfort	IBS Integra Biosciences
Vertical electrophoresis system Mini-PROTEAN®TetraCell	Bio-Rad
Vortex L46	Labinco
Water bath	Memmert

3.1.2 Disposables

Table 2: Disposables

Disposable/ Material	Company
6-well plates	Greiner Bio One
8-well CultureSlides	BD Bioscience
96-well plates U bottom	Nunc
Cell culture flasks 175 cm^3	Sarstedt
Cell strainer 70 µm	BD Bioscience
Chromatography paper Whatman™	GE Healthcare
Cover glasses, 24x 60 mm	Menzel-Gläser
FACS tubes 5 ml	BD Bioscience
Eppendorf tubes 0.5 ml, 1.5 ml, 2.0 ml	Eppendorf
Falcon tubes 15 ml, 50 ml	Greiner Bio-One
Filter V50 0,2µm	Sarstedt
Macs LS Columns	Miltenyi
Needle Microlance 3	BD Bioscience
Nitrocellulose membrane	Bio-Rad
Nunc® Sealing tape for multiwell plates	Sigma-Aldrich
Perfusor® syringe, 50 ml	B. Braun
Petri dish 35 x 10	Nunc

Pipette tips 1000, 200, and 10 µl	Starlab
Plastic pipettes 25 ml, 10 ml, 5 ml	Sarstedt
Plastic cuvettes	Sarstedt
Polypropylene tubes, 1.3 ml	Greiner
Pre-separation filters 30 µm	Miltenyi
Reaction cups 2 ml, 1.5 ml and 0.5 ml	Eppendorf
Syringe 1 ml Omnifix 40 solo	B. Braun
TechnoCut scalpel	HMD Healthcare
Tube, round bottom, 1.3 ml	Greiner
Tube, thinwall, ultra clear 38.5 ml	Beckman Coulter
UpCell Surface cell culture dish 92 x 17 mm	Nunc

3.1.3 Reagents

Table 3: Reagents

Reagent	Company
4',6-diamidino-2-phenylindole (DAPI)	Sigma-Aldrich
7x Protease inhibitor cocktail	Roche
Acetic acid	Merck
Acetone	Roth
Acrylamide/ Bis-acrylamide, 30 % solution	Bio-Rad
Ammonium chloride	Merk
Ammonium persulfate	Gibco
BGG protein standards 0.125 – 1.5 mg/ml	Fermentas
Bio-Rad protein assay dye	Bio-Rad
Boric acid	Roth
Bovine serum albumin	Roth
Bradford Reagent	Bio-Rad
Bromophenol blue	Sigma-Aldrich
BSA Standards, ready to use	Fermentas
Calcium chloride ($CaCl_2$)	Roth
CFDA-SE	Invitrogen
Citric acid	Roth
Coomassie Brilliant Blue (triphenylmethane)	Bio-Rad
D(+) saccharose	Roth
DMSO (Dimethyl sulfoxide)	Sigma-Aldrich
DTT (Dithiothreitol)	Roth
Ethanol	Roth
EDTA (Ethylenediaminetetraacetic acid)	Roth
FACS Clean solution	BD Bioscience
FACS Shutdown Solution	BD Bioscience
Fluorescence Mounting Medium	Dako
Glycine	Merck
Glutaraldehyde	Electron Microscopy Science

Hydrochloric acid (HCl)	Roth
Hydrogen peroxide (H_2O_2)	Roth
IAA (indole-3-acetic acid)	Sigma
Luminol	Sigma
Magnesium chloride ($MgCl_2$)	Merck
Methanol	Merck
Non-fat dry milk (NFDM)	Bio-Rad
NP-40	USB
NuPAGE Bis-Tris Gel 4-12%	Invitrogen
Ovalbumin grade V	Sigma
Ovalbumin purified	Worthington
Ovalbumin conjugate FITC	Molecular probes
p-Coumaric acid	Sigma
Paraformaldehyde	Roth
Ponceau S	Sigma-Aldrich
Potassium chloride (KCl)	Roth
Potassium hydroxide (KOH)	Merck
Potassium dihydrogen phosphate (KH_2PO_4)	Merck
Propidium iodide	Roth
Restore Western Blot Stripping Buffer	Thermo Scientific
Sodium azide (NaN_3)	Merck
Sodium chloride (NaCl)	Merck
Sodium hydrogen carbonate	Roth
Sodium hydrogen phosphate	Roth
Sodium n-dodecyl sulfate (SDS)	Calbiochem
Stripping buffer	Thermo Scientific
Sulfuric acid (H_2SO_4)	Roth
Tetramethylethylbenzidine (TMB)	Roth
Tetramethylethylenediamine (TEMED)	Merck
TRIS	Roth
TRIS-HCl	Roth
Triton X-100	Merck
Trypan blue	Sigma
Trypsin-EDTA 0.05%/0.02%	Roth
Trypsin-Inhibitor	
Tween-20	Roth
Uranyl acetate	Sigma

3.1.4 Enzymes

Table 4: Enzymes

Enzym	Company
Collagenase D	Roche
DNAse	Roche

3.1.5 Cytokines

Table 5: Cytokines

Cytokine	Concentration used	Company
IFNγ	50 ng/ml	BioLegend
IL-2	10 ng/ml	BioLegend
IL-4	10 ng/ml	BioLegend
IL-13	50 ng/ml	Immunotools
IL-17	10 ng/ml	Immunotools
IL-22	50 ng/ml	BioLegend
TNFα	25 ng/ml	Immunotools

3.1.6 Antibodies directed against murine antigens

Table 6: Antibodies

Antibody	Concentration used	Company
CD3ε	1 µg/ml	eBioscience
CD4 (L3T4), MicroBeads	10 µl/10^7cells	Miltenyi
CD8a (Ly-2), Micro Beads	10 µl/10^7cells	Miltenyi
CD28	1 µg/ml	eBioscience

Antibodies used in flow cytometry, fluorescence and confocal microscopy, and Western blot are stated within the corresponding section.

3.1.7 Size markers

Table 7: Size markers

Size marker	Company
PageRuler Prestained Protein Ladder	Thermo Scientific

3.1.8 Software

Table 8: Software

Program	Company
3 D -Software Imaris Bitplane Version 7.40	Bitplane
Adobe Illustrator	Adobe
Axiovision Rel 4.7	Zeiss
FACS DIVA software	BD Bioscience

LAS2000 Imaging System	Fujifilm
Multi Gauge V3.2	Fujifilm
Metamorph 6.3r2	Molecular Devices
Microsoft Office 2007	Microsoft
SAS software	SAS Institute

3.1.9 Commercial kits

Table 9: Commercial kits

Commercial kit	Application	Company
PKH-linker kit	fluorescent cell labeling	Sigma-Aldrich
murine ELISA kits: IFNγ, IL-6, IL-10, IL-12 p70	chemokines measurement	BioLegend

3.1.10 Buffers and solutions

Digest buffer (50 ml)

0.5 ml	1 M 2-(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
5 ml	1.5 M NaCl
0.5 ml	500 mM KCl
0.5 ml	100 mM $MgCl_2$
0.5 ml	200 mM Ca Cl_2
ad 50 ml	aqua
0.25 ml	1% DNAse
0.25 ml	10 mg/ml collagenase D

ECL 1 (10 ml)

1 ml	1 M Tris-HCl pH 8.5
9 ml	aqua
45 µl	90 mM coumaric acid
100 µl	250 mM luminol

ECL 2 (10 ml)

1 ml	1 M Tris-HCl pH 8.5
9 ml	aqua
6 µl	30% hydrogen peroxide

Erythrocyte lysis buffer 10x (1 l)

80.2 g	NH_4Cl
8.4 g	$NaHCO_3$
20 ml	0.5 M EDTA pH 8,0
ad 1 l	aqua

MACS buffer (500 ml)

495.5 ml	PBS
2.5 ml	FCS
2 ml	0.5 M EDTA pH 8.0

PBS 10x (5 l)

400 g	NaCl
10 g	KCl
72 g	Na_2HPO_4 x $2H_20$
10 g	KH_2PO_4
ad 5 l	aqua
adjust pH to 7.2	

Ponceau-S staining solution (50 ml)

50 mg	Ponceau-S
2.5 ml	acetic acid
ad 50 ml	aqua

Protein lysis buffer (20 ml)

200 µl	1 M Tris-HCl pH 7,0
1 ml	3 M sodium chloride
100 µl	NP40
ad 20 ml	aqua
1x	protease inhibitor cocktail

Running buffer 10x (2 l)

288 g	glycine
60 g	tris
100 ml	10% SDS solution
ad 2 l	aqua

Substrate buffer (11 ml)

10,89 ml	200 mM citric acid monohydrate
110 µl	30% hydrogen peroxide
5,1 µl	tetramethylbenzidine (TMB)

TBS-T (1 l)

10 ml	1 M TRIS/HCl pH 7,0
2 ml	0.5 M EDTA pH 8
50 ml	NaCl
500 µl	Tween 20 (T)
ad 1 l	aqua

Transfer buffer (1 l)

2.93 g	glycine
5.82 g	tris
3.75 ml	10% SDS solution
200 ml	methanol
ad 1 l	aqua

3.1.11 Cell culture reagents and media

Table 10: Cell culture reagents and media

Reagents	Company
β-mercaptoethanol	Sigma-Aldrich
FCS (fetal calf serum)	PAA
Hank's balanced salt solution (HBSS) without phenol red	Life technologies
2-(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	PAA
GM-CSF	Lab
L-glutamine	Invitrogen
non-essential amino acid	Invitrogen
PBS (phosphate buffered saline)	PAA
PCT Epidermal Keratinocyte Medium, CnT-07	CellnTec
Penicillin/ streptomycin (100x)	Invitrogen
RMPI 1640 with L-glutamine	Invitrogen

BMDC culture medium (500 ml)

440 ml	RMPI 1640 with L-glutamine
500 µl	50 mM β-mercaptoethanol
50 ml	fetal calf serum (FCS)
5 ml	200 mM L-glutamine
5 ml	100 U penicillin + 100 µg/ml streptomycin

T cell culture medium (500 ml)

476 ml	BMDC culture medium
12.5 ml	1 M 2-(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
5.6 ml	100 mM non-essential amino acid (Invitrogen)
5.6 ml	10 mM sodium pyruvate (Invitrogen)

MPEK culture medium (500 ml)

500 ml	PCT Epidermal Keratinocyte Medium, CnT-07
1x	supplement A
1x	supplement B
1x	supplement C

3.1.12 Animals

Table 11: Animals

Mouse	Company
C57BL/6	Charles River Laboratories
OT-I	Jackson Laboratory
OT-II	Jackson Laboratory

3.1.13 Long-term culture cells

Table 12: Long-term culture cells

Cell line	Company
murine epidermal keratinocyte progenitor cell line MPEK-BL6	CellnTec

3.2 Methods

3.2.1 Animals

Primary cells were isolated from C57BL/6, OT-I, or OT-II mice, respectively. Experiments were conducted according to the guidelines of the respective authority (LAVES, Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit). Mice were raised in the animal facility of the University Medical Center Göttingen under high hygienic levels, 12 h/12 h day/night cycle, and according to the natural needs of the species with food and water *ad libitum.*

3.2.2 Cell culture (primary and long term)

Cells were cultured under sterile conditions at 37°C, 5% CO_2, and 95% humidity. For long-term culture, the murine keratinocyte cell line MPEK-BL6 was used. As primary cells bone marrow-derived dendritic cells and CD4- and CD8-positive T cells were isolated.

3.2.2.1 Bone marrow–derived dendritic cells (BMDC)

Generation of BMDC was accomplished as described previously (Braun *et al.* 2010; Lutz *et al.* 1999). On day 0, bone marrow of tibia and femur from C57Bl/6 mice was rinsed out and cell suspension was collected. Erythrocytes were removed using erythrocyte lysis buffer for 7 min at room temperature. The remaining cells were washed twice with medium and counted with the help of a Neubauer counting chamber using trypan blue exclusion to determine cell viability. 2×10^6 of gained cells were sewed per tissue culture dish with 10 ml of BMDC culture medium in the presence of 10% (vol/vol) murine granulocyte macrophage colony-stimulating factor (GM-CSF)-containing supernatant. GM-CSF derived from a murine GM-CSF clone (hypoxanthine-aminopterin-thymidine-sensitive Ag8653 myeloma cell line transfected with murine GM-CSF cDNA isolated from a T cell clone by PCR and inserted into the vector BCMGSNeo) (Zal *et al.* 1994). On day 3 and 6, culture medium and GM-CSF were additionally added or used for replacement, respectively, to a total volume of 20 ml culture medium containing 10% (vol/vol) GM-CSF. On day 8, generation of BMDC was completed and non-adherent and loosely adherent cells, corresponding to the generated population of BMDC, were harvested and subjected to further experiments. Generated BMDC were considered as semi-mature.

3.2.2.2 Purification of CD4- and CD8-positive T cells

To assess the antigen-specific immunostimulatory potential of exosomes, CD4- and CD8-positive T cells from transgenic mice (OT-I and OT-II, respectively), expressing an OVA specific T cell receptor, were used. For T cell purification, spleen and axial, brachial, and inguinal lymph nodes were mechanically and chemically prepared. Shortly, spleen cells were passed through a 70 µm cell strainer, the cell suspension was depleted of erythrocytes using erythrocyte lysis buffer for 7 min at room temperature, and cells were washed in MACS buffer. Lymph nodes were chemical digested with digest buffer for 30 min at 37°C. Afterwards, cells were washed and CD4- and CD8-positive T cells of spleen and

lymph nodes were enriched using specific antibody-coated magnetic microbeads. The principle used was based on positive magnetic sorting (MACS). Desired cell population was labeled with magnetic antibodies. For labeling 90 µl of MACS buffer per 10^7 cells were incubated with 10 µl either CD4-or CD8a-specific micro beads per 10^7 cells for 15 min at 4°C in the dark. Suspension was washed with 1 ml MACS buffer per 10^7 cells. Cells were pelleted and collected in 1 ml of degassed MACS buffer. A separation column was attached to a magnet and rinsed with 1 ml of degassed MACS buffer. Afterwards, cell suspension was given through the column and the magnetic labeled cells were caught within the column whereas all other cells were rinsed out with 9 ml of MACS buffer and collected as negative population. Removal of the column from the magnet and rinsing of the column freed positive cells. CD4- and CD-8 positive T cells, respectively, were counted with the aid of a Neubauer counting chamber using trypan blue dye to determine cell viability.

3.2.2.3 Long term culture

The murine epidermal keratinocyte progenitor cell line MPEK-BL6 was cultivated in serum-free keratinocyte medium. Cells were cultivated in 175 cm² culture flasks and passaged when a confluence of 80-90% was reached to avoid differentiation of cells. For harvesting MPEK were washed with PBS and incubated with 5 ml 0.05%/0.02% trypsin-EDTA for 19 min at 37°C. To end the reaction, 5 ml 1% trypsin-inhibitor were added. MPEK were washed, pelleted and counted using a Neubauer counting chamber and trypan blue exclusion to determine cell viability. Keratinocytes were seeded at a density of 4.6-8.6 x 10^3 cells/cm^2 depending on the following experiments.

To demonstrate antigen uptake by MPEK, the model antigen OVA was used. Cells were incubated for 24 h with 10 µg/ml or 20 µg/ml of FITC labeled OVA. To distinguish between nonspecific attachment and active antigen uptake, cells were either incubated at 4°C or at 37°C, respectively. Analysis of fluorescence intensity was conducted by flow cytometry and immunofluorescence.

For exosome purification, MPEK were seeded at a density of 4.6 x 10^3 cells/cm^2 and cultured for a total of 96 h. Within the first 48 h, cells were incubated with or without 1 mg/ml OVA. To remove residual OVA from the supernatant, cells were then washed extensively with PBS. Thereafter, cells were stimulated for 48 h with or without 50 ng/ml IFNγ, or with 10 ng/ml IL-4 and 50 ng/ml IL-13, or with 10 ng/ml IL-17, 50 ng/ml IL-22, and 25 ng/ml TNFα. Afterwards, supernatant of MPEK was collected to purify exosomes, and MPEK were lysed for Western blot analysis. The different steps of incubation and stimulation described led to six different conditions as summarized in table 13. Differently generated exosomes were used for analysis and functional experiments.

Table 13: Conditions for exosome generation

0-24 h	24-48 h
+ medium	+ medium
+ medium	+ IFNγ
+ OVA	+ medium
+ OVA	+ IFNγ
+ medium	+ IL-4 IL-13
+ medium	+ IL-17, IL-22, TNFα

3.2.3 Exosome purification

To purify exosomes, cell culture supernatant of MPEK, raised under different conditions as described above, was collected. To remove remaining cells, supernatant was centrifuged at 2,000 x g. To eliminate cell debris, the supernatant was transferred to ultracentrifugation tubes and centrifuged at 10,000 x g. To remove further impurities, the supernatant was filtrated through a 0.2 µm pore filter and exosomes were pelleted at 100,000 x g for 2.5 h. The invisible pellet was either collected in 50 µl PBS per tube for the following experiments or lysed with NP40 containing lysis buffer for Western blot analysis. All ultracentrifugation steps were carried out at 4°C.

3.2.3.1 Density analysis of MPEK-derived membrane vesicles

To assess whether MPEK-derived membrane vesicles had a compatible density for exosomes, vesicles were layered on a continuous sucrose gradient. This method allows to separate membrane vesicles according to their density (Thery *et al.* 2006). The used gradient consisted of eight fractions carefully overlaid with 2.5-0.5 M sucrose in 20 mM HEPES. Membrane vesicles pelleted at 100,000 x g were collected in PBS and added upon the lightest fraction. Gradients were centrifuged for 15 h at 100,000 x g using a swinging bucket rotor. Thereafter, each fraction was diluted 1:8 in PBS and centrifuged again at 100,000 x g for 1 h. Pelleted membrane vesicles were collected in NP40 containing lysis buffer and subjected to Western blot analysis.

3.2.3.2 Fluorescence labeling of exosomes

To visualize aggregates of exosomes in fluorescence microscopy, exosomes were stained with lipophilic dye using a commercial linker kit for general membrane labeling. 100 µl of the exosome suspension were diluted in 900 µl Diluent C and mixed carefully. 1 µM PKH26 was added and exosomes were incubated for 5 min at room temperature. The reaction was stopped using 200 µl of exosome-free FCS. Afterwards, exosomal suspensions were pooled and diluted 1:6 in PBS to clear exosomes from redundant dye to avoid unspecific overspill. Exosomes were pelleted at 100,000 x g for 1 h.

3.2.4 Functional experiments

3.2.4.1 Co-culture of BMDC and exosomes

Generated BMDC were harvested on day 8 and extensively washed with culture medium containing exosome-depleted FCS. 2×10^6 cells per well of a six well culture dish were incubated in 2 ml exosome-depleted medium with 10 µg/ml MPEK-derived exosomes. BMDC were incubated with PKH26-labeled exosomes

to visualize exosome-uptake in a time series using immunofluorescence microscopy and flow cytometry. Cells were harvested after 0.5 h, 4 h, and 24 h.

For analysis of exosomes' impact on BMDC phenotype and interleukin profile, cells were incubated for 24 h with 10 µg/ml of non-labeled exosomes. Thereafter, BMDC were subjected to flow cytometry for phenotype analysis, and supernatant was collected for ELISA-based measurement of interleukin production. To determine if exosomes generated under various conditions differ regarding their impact on BMDC, the effect of different kinds of exosomes (Exo +/- OVA +/- IFNγ) on BMDC was analyzed. OVA-incubated (100 µg/ml) and medium only incubated BMDC served as controls.

3.2.4.2 T cell proliferation assay

For proliferation assays, CD4- or CD8-positive T cells were labeled with CFDA-SE. Intracellular; CFDA-SE is converted to the fluorophore CFSE. As CFSE binds to intracellular molecules and is present within the cytoplasm, every cell division will halve CFSE fluorescence intensity. T cells were incubated in 2.5 µM CFSE in PBS for 8 min at 37°C in the dark, followed by two washing steps with T cell medium to remove residual dye. 1 x 10^5 T cells in 200 µl medium were co-cultured in 96-well plates with either exosomes (10 µg/ml) or with exosome-pretreated BMDC in different T cell:BMDC ratios of 3:1, 10:1, or 30:1. Culture medium was used with or without IL-2 (10 ng/ml) as additional survival factor. Medium-incubated T cells and medium-incubated BMDC served as negative controls. T cells stimulated with CD3ε- and CD28-directed antibodies (1 µg/ml, respectively) and OVA-pretreated BMDC served as positive controls. After 72 h of co-incubation, cells were collected and measured by flow cytometry for CFSE intensity and supernatant was assessed by ELISA for IFNγ production.

3.2.5 ELISA

Cytokine production was quantified by a sandwich ELISA. Cell culture supernatant of BMDC or T cells was collected and measured for murine IL-6, IL-10, IL-12p70, or IFNγ, respectively, as recommended by the manufacturer. Briefly, on the first day 96-well plates were coated with 100 µl/well capture antibody dissolved in either coating buffer A as provided within the kit or with regard to IL-10 in 200 mM sodium dihydrogen phosphate. Plates were sealed and incubated at 4°C over night. After plates had been washed in 0.005% Tween in PBS, wells were incubated for 2 h at room temperature with 200 µl/well blocking buffer to prevent unspecific bindings. After three washing steps in 0.005% Tween in PBS, triplets of 100 µl per sample were loaded onto the different wells. Dilution of samples had been determined experimentally. Standard proteins as references to extrapolate concentration of chemokines were used as described by the company. After 2 h of incubation and three following washing steps in 0.005% Tween in PBS, plates were incubated for 1 h with 100 µl/well of detection antibody. Afterwards, plates were washed again as described above and avidin-horseradish peroxidase (HRP) was given to the samples for 30 min followed by five washing steps in 0.005% Tween in PBS. Subsequent 100 µl/well substrate buffer were added. Incubation was carried out in the dark, the duration depended onto the chemokines and lasted between 5 min (IL-6) and 15 min (IL-10). To end the reaction, 2 M sulfuric acid was added. The color change was measured at 450 nm in an Appliskan micro plate reader. To assure equal distribution of different solutions, plates were put onto a shaker during incubation.

3.2.6 Flow cytometry

Flow cytometry allows differentiation between cells due to their refraction of light. Additionally, cells can be labeled with fluorescence-coupled antibodies to analyze expression of proteins. Cells were harvested, washed in PBS, and pipetted into the tubes. 500,000 BMDC or MPEK and 100,000 T cells, respectively, were used per tube. To exclude unspecific binding, cells were washed in PBS and incubated with

10 µl anti-CD16/CD32 per sample for 10 min at 4°C. Thereafter, cells were stained with 10 µl of the desired antibody or the corresponding isotype control for 30 min at 4°C (for concentrations compare tab. 14), followed by another washing step. Immediately prior to measurement, 10 µl of 50 µg/ml propidium iodide (PI) were added to each sample to determine cell viability. Cells were analyzed with a FACS Canto II and the FACS DIVA software. Analysis of MPEK in flow cytometry could be accomplished without previous staining since OVA was directly labeled with FITC. In this case only additional staining with PI was accomplished. Phenotypic characterization of BMDC surface markers was performed on day nine with the following antibodies: CD11c, CD40, CD86, MHC I, and MHC II. When uptake of exosomes was analyzed, all markers except MHC I were measured. The phycoerythrin (PE) filter was suitable for PKH26 measurement for PKH-labeled exosomes. Analysis of T cells after co-culture with BMDC was conducted with antibodies directed against the following antigens: CD11c and CD4 or CD8a.

Table 14: Antibodies used in FACS

Antibody	Clone	Concentration used	Fluorophore
CD4	GK1.5	2 µg/ml	PE/Cy7
CD8a	53-6.7	2 µg/ml	PE/Cy7
CD11c	N418	2 µg/ml	APC/Cy7
CD16/CD32	2.4G2	5 µg/ml	purified
CD40	3/23	4 µg/ml	APC
CD86	GL-1	1 µg/ml	PE/Cy7
MHC I (H-2K^b)	AF6-88.5	2 µg/ml	PE
MHC II (I-A/I-E)	M5/114.152	5 µg/ml	FITC

With regard to all experiments, corresponding isotype controls were purchased from the same manufacturer and handled in the same manner as the specific antibodies (all BioLegend with the exception of anti-CD16/CD32 being purchased from BD Bioscience).

3.2.7 Microscopy

3.2.7.1 Fluorescence microscopy

For fluorescence microscopy analysis, MPEK were plated onto chamber slides and cultivated for 48 h followed by 24 h of incubation with 20 µg/ml FITC-labeled OVA. Incubation was carried out at different temperatures to differentiate between unspecific binding at 4°C and active antigen uptake at 37°C. Following, cells were extensively washed in PBS and fixed with ice-cold methanol for 10 min. BMDC, incubated with 10 µg/ml PKH-labeld exosomes for 24 h, were harvested, washed and prepared as cytospin preparation with a density of 50,000 cells/slide. Briefly, 100 µl of BMDC (500,000 cells/ml) were added per sample and centrifuged in cytospin for 10 min at 800 rpm. Dried slides were fixed in ice-cold methanol and let dry again. BMDC as well as MPEK were covered with Fluorescence Mounting Medium supplemented with 0.5 µg/ml DAPI for nuclear staining. An Axiovert 40 C microscope and AxioVision 4.7 software were used for image acquisition.

3.2.7.2 Confocal microscopy

To confirm OVA uptake by MPEK, confocal images were acquired. MPEK were cultivated in chamber slides and incubated with medium or purified OVA (1 mg/ml) for 24 h. For membrane labeling, cells were incubated for 10 min at 37°C with 300 µl/chamber of 3.3 µg/ml wheat germ agglutinin (WGA) Alexa Fluor 633 conjugate in HBSS. WGA labels membranes by selective binding to N-acetylglucosamine and N-acetylneuraminic acid residues (Wright 1984). After five washing steps with HBSS, keratinocytes were fixed in 4% PFA for 15 min at 4°C and were washed again in HBSS. To prevent antibodies from unspecific binding, samples were incubated with 5% FCS in PBS for 1 h. To facilitate penetration of antibodies through cell membranes, the blocking solution also contained 0.2% of Triton X to increase permeability. Anti-ovalbumin in 2.5% FCS served as primary antibody. Incubation was carried out overnight at 4°C. Afterwards, samples were extensively washed with PBS over 30 min before

incubation with secondary antibody in 2.5% FCS was conducted for 1 h at room temperature, followed by multiple washing steps in PBS. Samples were covered with Fluorescence Mounting Medium supplemented with 0.5 µg/ml DAPI for nuclear staining. Analysis was carried out with an Olympus Fluoview 1000 and the help of Dr. J. Wessels and J. Mahrt (MOLCI (Molecular and Live Cell Imaging service facility), University Medical Center Göttingen).

Table 15: Antibodies used in confocal microscopy

Antibody/ Lectin	Host	Concentration used	Fluorophore	Manufacturer
ovalbumin	mouse	1 µg/ml	purified	Biozol
mouse	goat	2.5 µg/ml	Alexa Fluor 488	Cell Signaling
WGA	--	3.3 µg/ml	Alexa Fluor 633	Invitrogen

3.2.7.3 Electron microscopy

Due to the size of exosomes of 50-100 nm (Thery *et al.* 2009), electron microscopy is necessary to visualize the vesicles. For analysis, the drop-to-drop method was used. Briefly, a carbon-coated 400-mesh copper grid was positioned on a drop of a liquid specimen to absorb for 1 min; excess fluid was carefully removed with filter paper. Following, the grid was placed within a large drop of 1% glutaraldehyde to absorb for 1 min, and excess fluid was once again removed. After washing in 5 drops of dH_2O, staining with 1% uranyl acetate for 30 sec was performed. The dried grid was examined with a CM120 electron microscope and images were recorded using an iTEM-Camera. D. Wenzel, Electron Microscopy Group, Max Planck Institute for Biophysical Chemistry, Göttingen, carried out preparation of samples and microscopy.

3.2.8 Protein analysis

3.2.8.1 Bradford assay

Proteins were quantified using Bradford assays. Coomassie Brilliant Blue binds to the present protein in the particular sample, the formed protein complex leads to a

change of the absorption maximum from 465 nm to 595 nm that can be measured photometrically. Before analysis, samples were prepared as follows: MPEK and spleen cells, purified as described in "Purification of CD4- and CD8-positive T cells" (page 19), were lysed with the appropriate amount of lysis buffer, mechanically homogenized, and lysates were centrifuged. The supernatant was analyzed further and the pellet was discarded. Exosomes resuspended in lysis buffer were subjected to Bradford assays. One blank and six samples with defined protein amounts were used to generate a standard concentration graph. Samples were prepared as follows:

800 µl	aqua dest.
1 µl	lysat
200 µl	color reagent BioRad Protein Assay
1 ml	sample

Samples were vortexed and measurement was accomplished with a spectrometer at 595 nm within 5 min to 1 h due to samples' stability in that time period.

3.2.8.2 SDS-Page and Western blot

Before samples were loaded onto a 10% acrylamide gel and subjected to SDS-PAGE analysis, 5x SDS sample buffer and 1 M DTT were added with final concentrations of 1x and 50 mM, respectively. Equal protein amounts were loaded onto the gel.

1x stacking gel	
1.55 ml	aqua bidest.
0.25 ml	30% acrylamide mix
0.19 ml	1 M Tris HCl pH 6.8
15 µl	10% SDS
15 µl	10% APS
1.5 µl	TEMED

1x separation gel 10%	
2 ml	aqua bidest.
1.65 ml	30% acrylamide mix
1.25 ml	1.5 M Tris HCl pH 8.8
50 µl	10% SDS
50 µl	10% APS
7.5 µl	TEMED

A prestained protein ladder (5 µl) was used as size marker. Samples were separated at 200 V with the usage of 1x running buffer until the dye reached the lower boarder of the gel. Using the procedure of semi dry blot and transfer buffer for 40 min at 150 mA, samples were transferred to a nitrocellulose membrane.

Successful transfer of proteins was controlled by membrane staining with Ponceau-S. To prevent antibodies from unspecific binding, the membrane was blocked for 1 h at room temperature with 5% non-fat-dry-milk (NFDM) in TBS-T. Afterwards, incubation with primary antibodies occurred overnight at 4°C. The following antibodies were dissolved in 1% NFDM in TBS-T, with the exception of anti-alix, which was dissolved only in TBS-T:

Table 16: Primary antibodies used in Western blot analysis

Antibody	Host species	Concentration used	Company
actin	mouse	0.3 µg/ml	Millipore
alix	mouse	1 µg/ml	Biozol
calnexin	rabbit	1 µg/ml	antibodies-online
flotillin	mouse	50 ng/ml	BD Bioscience
lamin	rabbit	0.5 µg/ml	Cell Signaling
MHC class I H2 Kb	rabbit	5 µg/ml	Abcam
MHC II (I-A/I-E)	rat	10 µg/ml	Millipore
ovalbumin	mouse	0.2 µg/ml	Sigma-Aldrich

After three washing steps with TBS-T, membranes were incubated with HRP-coupled secondary antibodies in 0.83% NFDM in TBS-T for 1 h at room temperature. The following secondary antibodies were used:

Table 17: Secondary antibodies used in Western blot analysis

Antibody	Host species	Concentration used	Company
mouse	goat	4 µg/ml	Promega
rabbit	goat	4 µg/ml	Promega
rat	goat	0.67 µg/ml	Linaris

After three washing steps as described above, membranes were incubated with ECL solution 1 and 2 in a ratio of 1:1. For signal detection, the LAS 2000 imaging system was used. To confirm equal protein loading, membranes were washed thrice in TBS-T, stripped using commercial stripping buffer, washed thrice again and incubation with primary antibodies (actin, lamin) was carried out as described above.

3.2.8.3 Proteomic analysis

The content of MPEK-derived exosomes was assessed by proteomic analysis. Proteins of purified exosomes were separated by one-dimensional SDS-PAGE and the gel was stained with Coomassie Brilliant Blue. Slices of the cut gel were reduced with 10 mM DTT, alkylated with 55 mM IAA for 20 min at 26°C, and digested with trypsin overnight at 37°C (Shevchenko *et al.* 1996). Tryptic peptides were injected onto a C18 pre-column to elute bound peptides. Thereafter, bound peptides were separated on a C18 capillary column using a nanoflow LC system coupled to an LTY-Orbitrap XL hybrid mass spectrometer using MS and MS/MS acquisition alternately. Raw data were analyzed with Mascot search engine against NCBI protein database for peptide and protein identifications. For annotation of acquired data, Scaffold (Proteome Software, Version 3.4) was applied with the following parameter settings: minimum peptide and protein identification probability 80% and minimum of 2 identified peptides per protein. As basis for analysis, every sample had to express the exosomal markers flotillin, alix, and tsg101. To be considered to be expressed at higher or lower levels, the respective protein had to be twice as much or half in comparison to medium control. Proteomic analysis was carried out by Prof. H. Urlaub, Bioanalytical Mass Spectrometry Group, Max Planck Institute for Biophysical Chemistry and Bioanalytics, Göttingen.

3.2.9 Statistics

Statistic data analysis was done using the rank-sum test with ANOVA-type statistics (Brunner *et al.* 2001). Data are visualized as means with error bars indicating standard deviation (SD), * indicates $p < 0.05$.

4 Results

4.1 The murine kerationcyte cell line MPEK produces exosomes

To address the question whether murine keratinocytes are able to produce exosomes, the MPEK cell line was cultivated and analyzed using a modified standard protocol for exosome purification (Thery *et al.* 2006). Supernatant of MPEK that had been treated with or without IFNγ (to imitate inflammatory conditions) was collected and after different centrifugation and filtration steps purified membrane vesicles of MPEK were obtained. Of interest, the quantity of produced exosomes by MPEK was comparable with and without IFNγ stimulation (Appendix table 28). Exosomes were analyzed with regard to their main characteristics: their size of 50-100 nm and their saucer-like shape in electron microscopy, their expression of exosomal markers like alix and flotillin in Western blot and proteomic analysis (Thery *et al.* 2009), and their density of 1.10-1.21 g/ml in sucrose gradients (Mathivanan *et al.* 2012). MPEK-derived membrane vesicles pictured in electron microscopy had a saucer-like shape and a diameter of 60-90 nm (Fig. 5a). The presence of the exosomal markers flotillin and alix and the absence of calnexin, a protein of the endoplasmic reticulum indicating contamination (Thery *et al.* 2006), were demonstrated by Western blot analysis (Fig. 5b). Moreover, these findings, as well as the presence of additional exosomal markers like tsg101 and CD81, were confirmed by proteomic analysis (Simpson *et al.* 2008) (Table 18). For further characterization, membrane vesicles were layered on a continuous sucrose gradient to determine the vesicle density. Analysis of the different density fractions by Western blot revealed the presence of flotillin at a density of 1.10 g/ml (Fig. 5c), consistent with exosomal content. Thus, with regard to shape, size, density, and expression of exosomal markers, MPEK-derived membrane vesicles meet all required criteria and could be considered as exosomes.

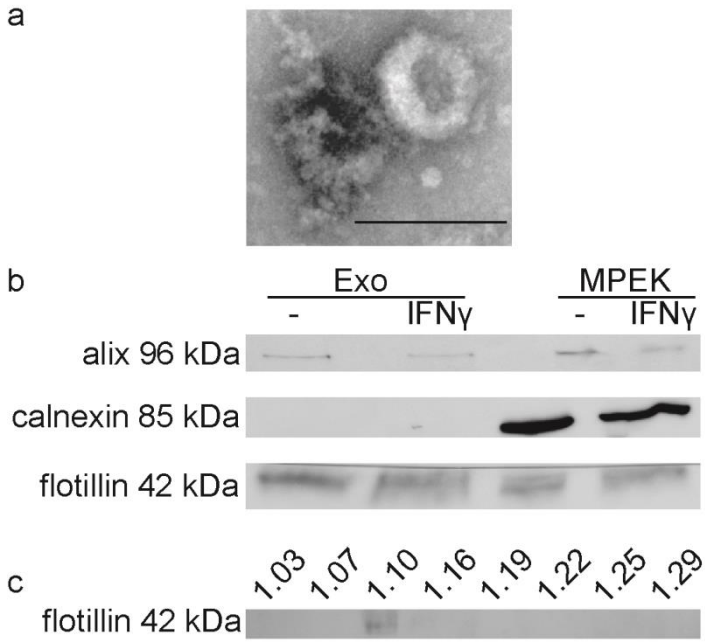

Figure 5: MPEK produce exosomes. Analysis of MPEK-derived membrane vesicles with regard to exosome-defining traits. (**a**) Purified vesicles were visualized by electron microscopy picturing their saucer-like shape and size between 60-90 nm. Scale bar = 100 nm. (**b**) The presence of the exosomal markers alix and flotillin as well as the absence of the negative marker calnexin was detected by Western blot. 30 µg of lysate per sample were used for analysis of calnexin and flotillin and 40 µg for analysis of alix. Flotillin also served as loading control for calnexin. (**c**) Density analysis of the purified vesicles by means of sucrose gradient revealed the presence of flotillin in the fraction of 1.10 g/ml, compatible with exosomal density.

Table 18: Exosomal markers detected by proteomic analysis

Protein name	Exosome	Exosome IFNγ	MW (kDa)	Accession no.
alix	+	+	96	NP_001158150
calnexin	--	--		
CD80	+	+	35	NP_033985
CD81	+	+	18	NP_598416
flotillin-1	+	+	48	NP_032053
tsg101	+	+	44	NP_068684

4.2 MPEK internalize antigen and shuttle it to their exosomes

To assess whether MPEK are able to take up antigen and transfer it to their exosomes, the model antigen ovalbumin (OVA) was used. Keratinocytes were incubated with fluorescence labeled OVA at 4°C or 37°C to distinguish between unspecific binding and active antigen uptake, respectively. As demonstrated by fluorescence microscopy (Fig. 6a) and FACS analysis (Fig. 6b), MPEK incubated at 37°C, unlike their counterparts incubated at 4°C, showed a strong fluorescence signal indicating active internalization of antigen. To assure antigen uptake and localization within the cell, OVA incubated MPEK were also analyzed by confocal microscopy confirming previous findings (Fig. 6c).

Furthermore, uptake of pure OVA protein pictured by Western blot showed a doublet band at around 45 kDa that was also present when OVA incubated MPEK were analyzed (Fig. 6d). As exosomes contain membrane and cytosolic proteins of their cell of origin (Simpson *et al.* 2008), OVA internalized by MPEK should be transferred to MPEK-derived exosomes. Confirming this hypothesis, exosomes purified from OVA incubated MPEK showed a single band at 45 kDa in Western blot (Fig. 6e). When MPEK were stimulated with IFNγ prior to exosome purification, the OVA signal in Western blot was enhanced (for quantification, in densitometric analysis enhancement was more than threefold), suggesting an increase of antigen uptake and transfer under inflammatory conditions.

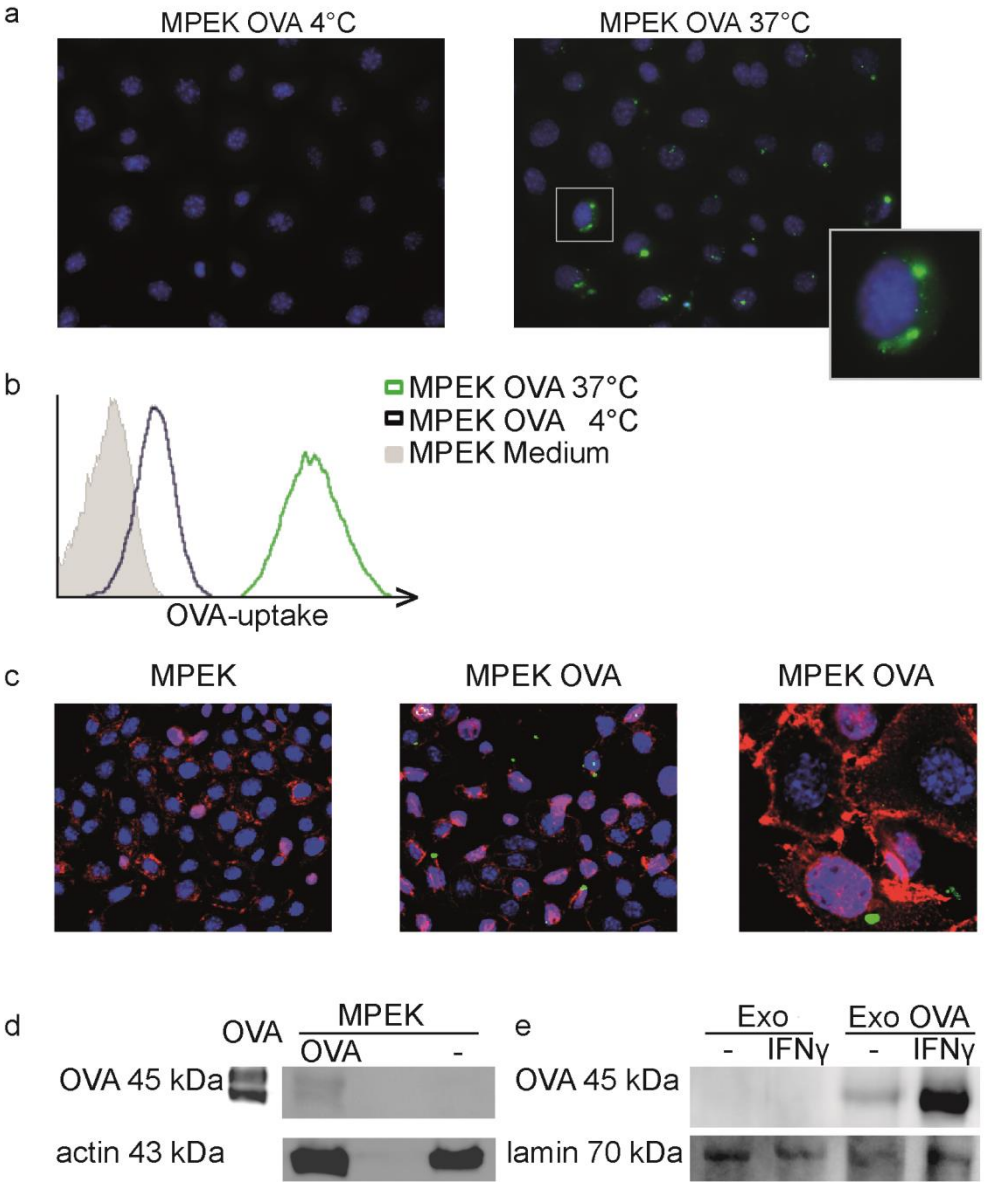

Figure 6: MPEK internalize OVA and shuttle it to their exosomes. To distinguish between active antigen uptake versus unspecific binding MPEK were incubated with FITC-labeled OVA (green) at 4°C and 37°C as pictured by (**a**) fluorescence microscopy and (**b**) flow cytometry, respectively. 20 µg/ml were used for analysis by fluorescence microscopy and 10 µg/ml for analysis by flow cytometry. In contrast to incubation at 4°C, incubation at 37°C led to a strong increase of fluorescence intensity suggesting active antigen uptake. For flow cytometry, only viable cells were taken into consideration. (**c**) Findings were confirmed by confocal microscopy demonstrating the presence of antigen (green) in OVA incubated MPEK. (**d**) Analysis by Western blot revealed the presence of OVA in lysates of antigen incubated MPEK, presenting the same doublet band as seen with pure OVA protein. 40 µg of MPEK lysate per sample were used. Actin served as loading control. (**e**) To demonstrate antigen transfer to MPEK exosomes, exosomes derived from OVA incubated MPEK were analyzed. Western blot demonstrates the presence of OVA in exosomes and even an enhanced signal intensity when additonal IFNγ stimulation of MPEK had occurred. 24 µg of lysate per exosome sample were used. Lamin served as loading control.

4.3 BMDC take up MPEK-derived exosomes with an increased quantity over time

Dendritic cells function as professional APC. Depending on their phenotype, immature dendritic cells efficiently take up antigen, whereas mature dendritic cells mainly present it (Banchereau and Steinman 1998). BMDC generated *in vitro* were considered immature or semi-mature, prior to incubation with exosomes. In line with previous publications for BMDC exposed to DC-derived exosomes (Morelli *et al.* 2004), BMDC were able to internalize MPEK-derived exosomes as demonstrated by flow cytometry and immunofluorescence using PKH26-labeled exosomes (Fig. 7a and b). The time series of 0.5 h, 4 h, and 24 h of incubation demonstrated an increase of exosome internalization over time, however, showing strong interindividual differences between single BMDC. Since exosomes are too small to be visualized as individual particles by fluorescence microscopy, images depict aggregates of exosomes in a typical granular pattern (Fig. 7c) (Lin *et al.* 2007), due to exosome accumulation in lysosomes after uptake (Tian *et al.* 2013).

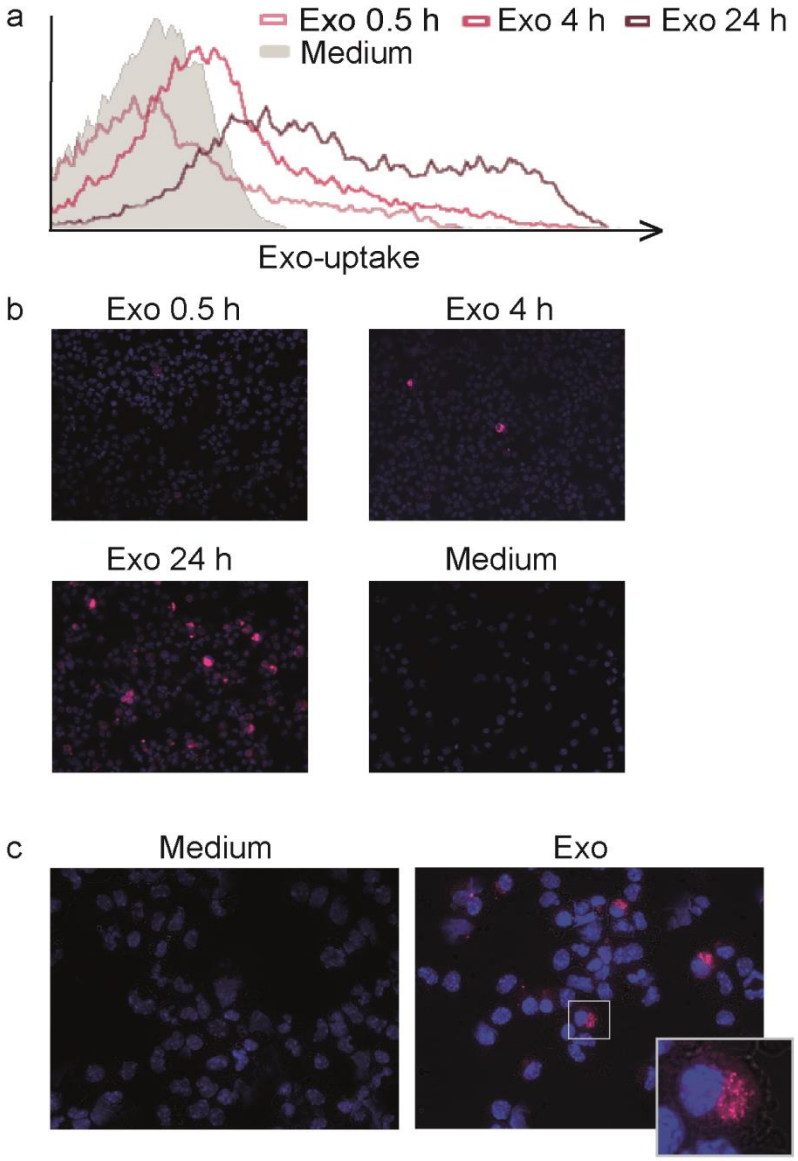

Figure 7: Uptake of MPEK-derived exosomes by BMDC increases over time. To visualize internalization of MPEK-derived exosomes by BMDC, BMDC had been incubated with 10 µg/ml PKH-labeled exosomes. Uptake of MPEK-derived exosomes was analyzed after 0.5 min, 4 h, and 24 h of incubation by (**a**) flow cytometry and (**b**) fluorescence microscopy, respectively. For flow cytometry only viable CD11c-positive cells were taken into consideration. (**c**) Amplification of BMDC in immunofluorescence after 4 h of incubation with exosomes revealed the typical granular aggregates visible in the cytoplasm. BMDC incubated with normal medium served as negative control.

4.4 Exosome-uptake enhances production of IL-6, IL-10, and IL-12 and CD40 expression by BMDC

As BMDC were considered semi-mature before exosome incubation, the influence of exosome uptake on maturation was examined. Toward this end, BMDC where incubated for 24 h with different exosomes (Exo +/- IFNγ +/- OVA) and phenotype and interleukin profile were analyzed *in vitro*. Exosome uptake induced BMDC to produce higher amounts of IL-6, IL-10, and IL-12 as measured by ELISA (Fig. 8a). Importantly, incubation of BMDC with medium or OVA as controls did not show any comparable effects. In line with immunostimulatory potential of exosomes (Skokos *et al.* 2003), uptake led to maturation of BMDC as demonstrated by a significantly enhanced expression of CD40 compared to medium control. However, exosome internalization was not able to influence expression of CD86, MHC I, or MHC II (Fig. 8b). Overall, MPEK-derived exosomes showed a general immunostimulatory effect on BMDC, whereas no significant differences between antigen containing and/or IFNγ stimulated exosomes could be observed.

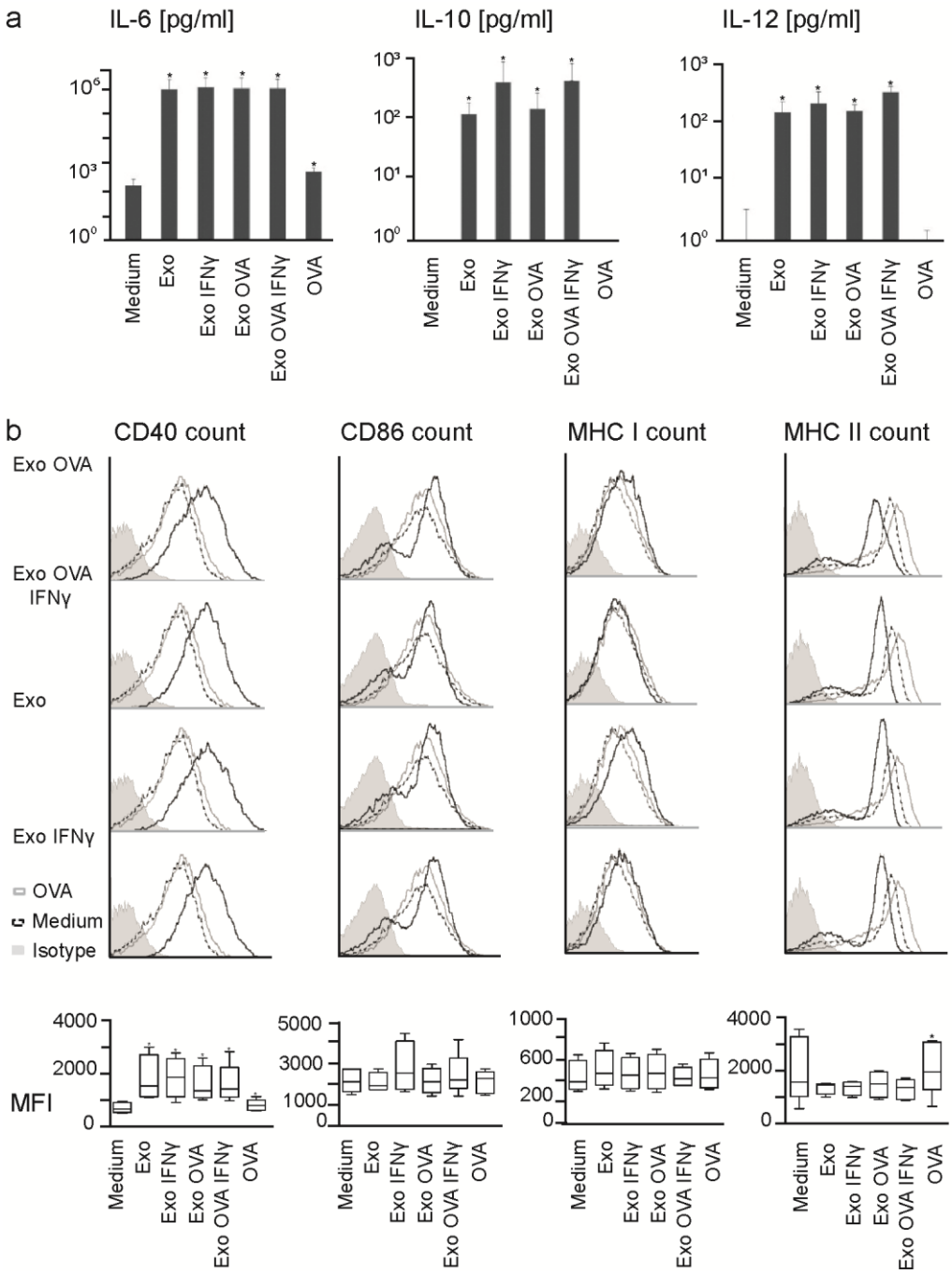

Figure 8: Exosome-loaded BMDC increase IL-6, IL-10, and IL-12 production and CD40 expression. To assess whether MPEK-derived exosomes have an impact on BMDC phenotype and interleukin profile, BMDC were incubated with 10 µg/ml of different loaded exosomes (Exo +/- OVA +/- IFNγ). Cytokine production and phenotype were analyzed by (**a**) ELISA and (**b**) flow cytometry, respectively. Interleukin production of IL-6, IL-10, and IL-12 was significantly enhanced, whereas with regard to phenotype only the expression of (**b**) CD40 but not of CD86, MHC I, or MHC II was increased. Only viable and CD11c-positive BMDC were analyzed phenotypically. There were no significant differences regarding the effects detected with different loaded exosomes. * indicates $p < 0.05$ compared to medium incubated BMDC. Statistical analysis was calculated with the rank-sum test with ANOVA-type statistic (Brunner *et al.* 2001), n = 5.

4.5 Antigen-containing exosomes fail to induce direct or BMDC-mediated antigen-specific T cell responses

To assess whether exosomes deliver OVA protein unbound or its processed peptide derivates bound to MHC molecules, exosomal expression of MHC I and II was investigated by Western blot and proteomic analysis (Fig. 9a, Table 19). Western blot displayed the presence of MHC I in IFNγ stimulated as well as control (medium incubated) MPEK, whereas for exosomes, MHC I could only be detected in exosomes derived from IFNγ stimulated MPEK. Proteomic analysis of Exo and Exo IFNγ confirmed those findings with detection of H-2 class I histocompatibility antigen, L-D alpha chain precursor in samples of Exo IFNγ. No MHC II molecules could be detected in any preparations, neither of MPEK nor in any of their exosomes. This finding was irrespective of IFNγ stimulation.

The question whether MPEK-derived exosomes would elicit antigen-specific T cell responses, was addressed by *in vitro* proliferation assays. Given that keratinocytes are non-professional APC, they express very few, if any, costimulatory molecules they could transfer to their exosomes (Nickoloff and Turka 1994). To compensate for this lack of molecules, co-culture experiments with BMDC were conducted. BMDC loaded with antigen-containing exosomes were incubated with CFSE stained CD4- or CD8-positive T cells derived from OT-I or OT-II mice, respectively. After a total of 72 h of co-culture, T cells were measured by flow cytometry for CFSE intensity and the supernatant was analyzed for IFNγ production (Fig. 9b and c). No significant changes of fluorescence or IFNγ production could be detected indicating that antigen-loaded exosomes failed to induce antigen-specific T cell responses. To test the hypothesis that exosomes could elicit T cell responses directly, antigen containing and, due to its pivotal role for immune responses, IFNγ stimulated exosomes (van Niel *et al.* 2003) were introduced into the same experimental set up and incubation with either CFSE stained CD4- or CD8-positive T cells was carried out (Fig. 9d). It was found that OVA-containing IFNγ stimulated

exosomes also failed to induce antigen-specific CD4 or CD8 T cell proliferation. All results were irrespective of additional T cell stimulation with IL-2.

Table 19: MHC expression in MPEK-derived exosomes

Protein name	Exosome	Exosome IFNγ	MW (kDa)	Accession no.
H-2 class I histocompatibility antigen, L-D alpha chain precursor	--	+	41	NP_034510
MHC II	--	--		

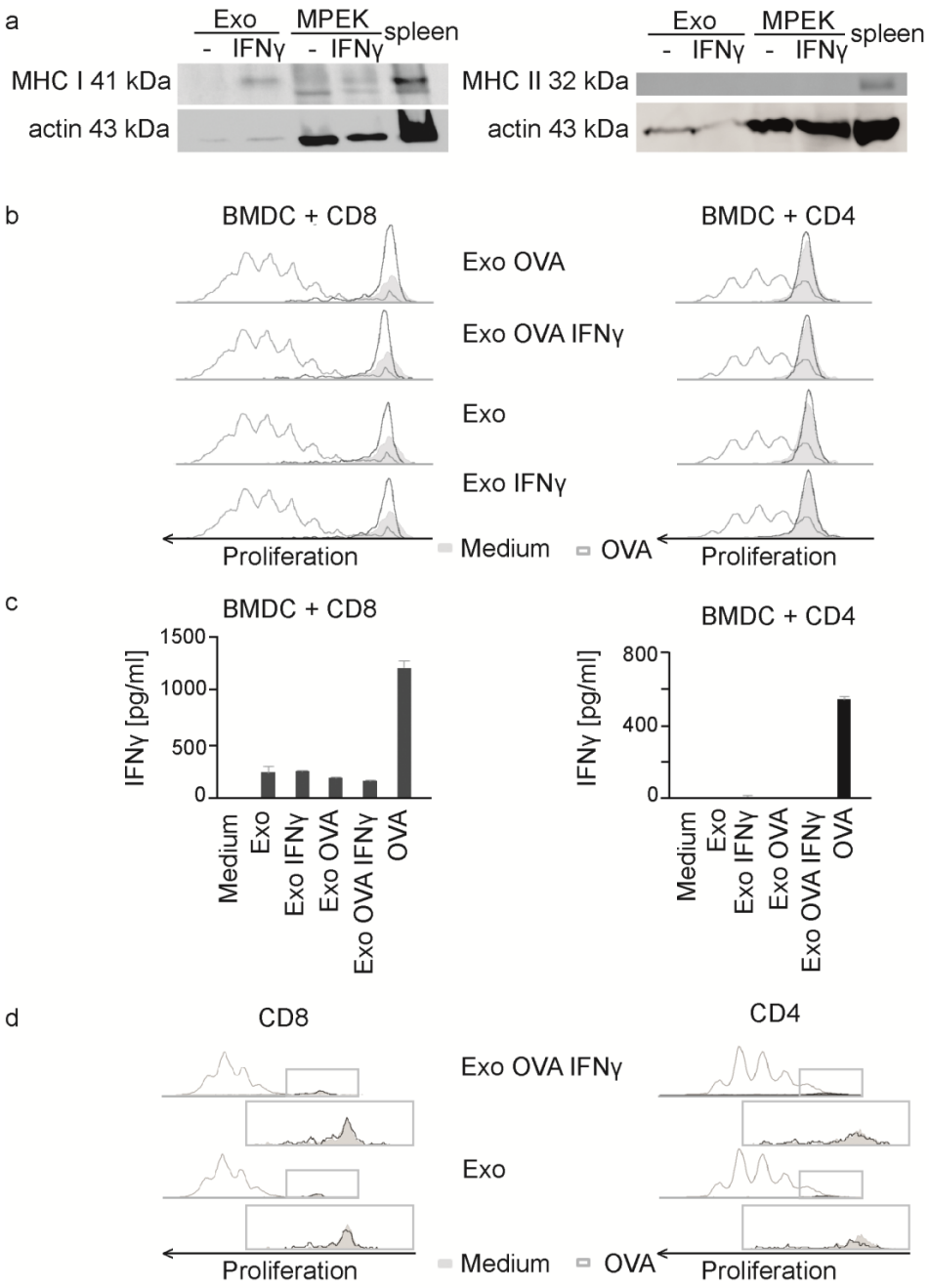

Figure 9: Antigen-loaded exosomes failed to induce antigen-specific T cell response directly or via BMDC. To analyze whether OVA protein is delivered unbound or bound to MHC molecules in exosomes, MHC I and MHC II expression of MPEK and their exosomes was analyzed by Western blot (**a**). Spleen lysate served as positive control. (**b**, **c**) For functional analysis, exosome-loaded BMDC were incubated for 72 h with antigen-specific T cells from OT-I or OT-II mice, respectively, and the T cell response was determined by (**b**) flow cytometry for proliferation and (**c**) by ELISA for IFNγ production. OVA-loaded BMDC and medium-incubated BMDC served as positive and negative controls, respectively. Analysis of a BMDC:T cell ratio of 1:3 is displayed. (**d**) Second, antigen-loaded IFNγ stimulated exosomes (10 µg/ml) were directly incubated with T cells, and proliferation was measured by flow cytometry. CD3/CD28 stimulated T cells and medium incubated T cells were used as positive and negative controls, respectively. For T cells to be taken into consideration, cells had to be viable, CD11c-negative, and CD4- or CD8-positive, respectively. Data are depicted as mean +/- standard deviation for triplicate cultures.

4.6 Composition of MPEK-derived exosomes is changed under defined conditions modeling clinical settings

As shown before, exosomes were able to affect BMDC phenotype and interleukin production. To address the question whether environmental conditions were able to affect exosome composition, as demonstrated for IFNγ stimulation and MHC expression, MPEK were cultured under different conditions and their exosomes were analyzed by proteomic analysis. Skin diseases are known to affect the cytokine profile of keratinocytes *in vivo*, as it has been studied in various mouse models (Schön 2008; Spergel *et al.* 1998). In this experimental set up the cytokine environment of chronic skin diseases was simulated *in vitro*. In addition to the inflammatory stimulus IFNγ, the frequent chronic diseases psoriasis and atopic dermatitis display distinctly different patterns of inflammatory mediators. Based on this notion, MPEK were either incubated with IL-17, IL-22, and TNF-α as a typical cytokine profile of psoriasis (Raychaudhuri 2012) or with IL-4 and IL-13 representative for atopic dermatitis (Bieber 2012). Exosomes generated under steady state conditions (medium) were analyzed for comparison. The following table (Table 20) presents an overview of proteins involved in adhesion, cytoskeleton, membrane fusion and transport, metabolism, antigen presentation, protein folding, and signaling. Proteins presented have been defined previously in exosomal preparations as presented in ExoCarta (Mathivanan and Simpson 2009).

The results suggest a specific accumulation of proteins depending on specific conditions. In comparison to exosomes generated under steady state, influence of the changed environmental cytokine profile could be observed in every protein family:

Table 20: Exosomal composition under different environmental conditions

	Exo					
	medium	**IL-17, IL-22, TNF-α**	**IL-4, IL-13**	**IFNγ**	**MW (kDa)**	**Accession no.**
Cell adhesion						
basal cell adhesion molecule	-	+	+	++	68	EDL23193
claudin-1	+	+	++	+	23	NP_057883
E-cadherine	-	-	-	+	98	P09803
integrin α-6	+	+	+	+	120	NP_032423
lactadherin/ milk fat globule-EGF factor 8	+	+	+	+	47	NP_032620
laminin B1	++++++	+	+++++	+++++	202	AAA39407
Cytoskeleton						
actin β	+	+	+	+	42	NP_001092
actinin α 1	+++	+	+++	+++	103	NP_598917
cofilin-1	+	+	+	+	19	NP_031713
filamin B	-	-	-	+	277	NP_598841
keratin 13 type I	+	-	-	-	48	NP_034792
Membrane fusion and transport						
alix/ programmed cell death 6-interacting protein	+	+	+	+	96	NP_035182
annexin A1	+	+	+	+	39	NP_034860
annexin A4	+	+	++	+++	36	NP_038499
cation-dependent mannose-6-phosphate receptor precursor	++	++	++	+	31	NP_034879
rab 10	+	+	+	+	23	NP_057885
rab 14	+	++	++	++	24	NP_080973
Metabolic enzymes						
fatty acid synthase	+++++	+	++++	++++	273	EDL34808
glucose-6-phosphate isomerase	+++	+	+++	+	63	NP_032181
glyceraldehyde-3-phosphate dehydrogenase	+	+	+	+	36	NP_032110
L-lactate dehydrogenase A chain	+	+	+(+)	+(+)	40	NP_034829
malate dehydrogenase 1, cytoplasmic	+	+	+	+	40	NP_032644
pyruvate kinase, muscle	+	+	+	+	58	NP_035229

	Exo					
	medium	**IL-17, IL-22, TNF-α**	**IL-4, IL-13**	**IFNγ**	**MW (kDa)**	**Accession no.**
MHC molecules						
H-2 class I histocompatibility antigen, L-D alpha chain	-	-	-	+	41	NP_034510
MHC II	-	-	-	-		
Protein folding						
calreticulin precursor	+	+	+	+	48	NP_031617
heat shock protein 90α	++++	+	++++	+++	85	NP_034610
heat shock protein 90β	+	+	+	+(+)	92	NP_032328
protein disulfide isomerase A3 precursor	+	+	+	+	57	NP_031978
Signaling						
14-3-3 η	++	+	++	+	28	NP_035868
14-3-3 σ	+	+	+	+	28	NP_061224
14-3-3 ε	+	+	+	+	29	NP_006752
guaninenucleotide-binding G(i) α2	+	+	+	+	40	NP_032164
major vault protein	++	+	++	++	97	NP_542369
phospholipase D3	-	-	+	-	54	NP_035246

5 Discussion

5.1 Characterization of MPEK-derived membrane vesicles

During the last decades, research on exosomes has focused on hematopoietic cells. Thus, little is known about epithelial cell-derived exosomes. In this work, it was demonstrated for the first time that murine keratinocytes produce exosomes under steady state as well as under inflammatory conditions (Figure 5 page 32). Before, exosome production had only been shown for human keratinocytes (Chavez-Muñoz *et al.* 2008) and other epithelial cells as intestinal epithelial cells (van Niel *et al.* 2001).

5.2 Immunological aspects of MPEK-derived exosomes

5.2.1 MPEK-derived exosomes contain antigenic information

Keratinocytes play a pivotal role in the cutaneous immune system. Besides their contribution to the innate immune system (Pivarcsi *et al.* 2005), they also take part in adaptive immune responses (Nickoloff and Turka 1994). To function as non-professional APC, keratinocytes have to take up antigen for presentation. Analysis by FACS and immunofluorescence microscopy demonstrated a strong increase of fluorescence when MPEK were incubated with FITC-labeled OVA at 37°C whereas no change could be detected at 4°C. This dependence on temperature strongly suggests active antigen uptake instead of unspecific binding. Confocal microscopy and Western blot confirmed these results by demonstrating the presence of OVA via antibody reaction within the cell or cell lysate (Figure 6 page 34). Furthermore, these findings are in line with previous publications demonstrating not only the uptake, but also effective processing and presentation of antigen by keratinocytes (Black *et al.* 2007). As mentioned previously, exosomes contain a specific protein repertoire consisting of cytosolic and membrane proteins of their mother cell with a specific enrichment of certain proteins (Ratajczak *et al.* 2006;

Simpson *et al.* 2008). As a component of this molecular pattern, antigen internalized by MPEK was also transferred to their exosomes. As expected (Blume *et al.* 2009), increased allergen uptake by keratinocytes occurs under IFNγ stimulation. Lysates of MPEK-derived exosomes showed that their exosomes contained a higher quantity of antigen when MPEK had not only been exposed to the respective antigen but had been stimulated with IFNγ as well (Figure 6 page 34).

5.2.2 Immunostimulatory effect of MPEK-derived exosomes on BMDC

Keratinocytes and DC, which are so-called Langerhans cells within the epidermis, maintain a close regional relationship to each other. In the experimental setting employed here, BMDC took up MPEK-derived exosomes as shown by fluorescence microscopy and FACS analysis (Figure 7 page 36). This finding is partially supported by previous publications, demonstrating BMDC to internalize DC-derived exosomes *in vitro* (Morelli *et al.* 2004). The uptake increased over time, with a strong inter-individual difference between particular cells (Figure 7 page 36), a notion that is possibly associated with different states of maturation (Banchereau and Steinman 1998; Morelli *et al.* 2004).

Keratinocytes are the most abundant cells within the skin. With their key role in the cutaneous immune system, they are able to directly kill or fight invading microbes through the production of antimicrobial peptides (AMP). However, they also link innate and adaptive immunity via AMP- and TLR-dependent activation of DC (Pivarcsi *et al.* 2005). Because keratinocytes are very numerous and are located adjacent to DC, it is conceivable that they “select” and transfer important information not only via AMP but also via their exosomes to regional DC, similar to the short range mechanism of exosomes of immature DC to spread alloantigen between DC (Montecalvo *et al.* 2008). This hypothesis is further supported by recent findings suggesting a specific uptake and target selection of exosomes via tetraspanins (Rana and Zoller 2011).

MPEK-derived exosomes were not only internalized by BMDC but their uptake also influenced BMDC phenotype and interleukin production, indicating an induction of maturation. DC maturation is crucial for the initiation of immune responses and is known to be induced by microbial products and certain cytokines as IL-1 or TNF-α *in vivo* (Banchereau and Steinman 1998). *In vitro*, internalized MPEK-derived exosomes induced BMDC to produce high amounts of IL-6, IL-10, and IL-12. Additionally, expression of the surface and maturation marker CD40 was highly increased by uptake of MPEK-derived exosomes, whereas CD86, MHC I, or MHC II expression was not affected (Figure 8 page 38). Other studies demonstrated mast cell-derived exosomes to increase not only CD40 expression of BMDC, but also CD86 and MHC II expression (Skokos *et al.* 2003). In contrast, blood-borne exosomes did not affect splenic DC phenotype at all (IA^b, CD54, CD86) (Morelli *et al.* 2004). Moreover, MPEK-derived exosomes altered the interleukin profile of BMDC. In recent experiments, a changed interleukin profile of macrophages through melanoma cell-derived exosomes has been demonstrated, which included induction of IL-8 and IL-1Ra but not of IL-6, IL-10, or IL-12. The observed cytokine profile could not be assigned in a strict sense to a TH1 or TH2 type response (Marton *et al.* 2012). Further, trophoblast-derived exosomes were able to upregulate chemokine production of monocytes (amongst others IL-1β, IL-6, and TNFα) (Atay *et al.* 2010). However, even though MPEK-derived exosomes did influence BMDC phenotype and interleukin profile, this effect was comparable between the different kinds of exosomes (Exo +/- OVA +/- IFNγ). These findings emphasize two major points: the influence of exosomes strongly depends on the cell type of origin and MPEK-derived exosomes seem to have a general but not a specific immunostimulatory effect. With regard to the more complex *in vivo* situation, DCs are exposed to a multitude of exosomes derived from different cell types. Thus, changes of ratio between the different kinds of exosomes might be more relevant for DC maturation than the kind of exosome itself.

5.2.3 Antigen-specific functions of MPEK-derived exosomes

MPEK-derived exosomes failed to induce an antigen-specific T cell response alone and via BMDC (Figure 9 page 41). This finding was somewhat surprising. Due to their shortage of co-stimulatory molecules only a couple of exosomes are able to elicit T cell responses directly (Admyre *et al.* 2006; Kovar *et al.* 2006). However, several studies have demonstrated that exosomes can stimulate T cells in the presence of APC (Andre *et al.* 2002; Andre *et al.* 2004).

The failure of MPEK-derived exosomes to induce T cell proliferation via BMDC could be due to the décor of co-stimulatory molecules. Most experiments have been conducted with immune cell-derived exosomes. Immune cells, in comparison to keratinocytes, function as professional instead of non-professional APC and constitutively express a higher abundance of MHC and co-stimulatory molecules (Chavez-Muñoz *et al.* 2009; Segura *et al.* 2005b). However, intestinal epithelial cell-derived exosomes elicited a humoral immune response even though these exosomes were free of any co-stimulatory molecules. This study also emphasized the pivotal role of IFNγ, since only exosomes derived from IFNγ-stimulated cells were effective (van Niel *et al.* 2003). In line with our findings that MPEK did not express MHC II under IFNγ stimulation, this failure of expression has also been demonstrated for other murine keratinocytes *in vitro* before (Gaspari and Katz 1988). However, transfer of MHC class II-restricted allopeptides to MHC class II molecules on the surface of APCs seems to be required (Montecalvo *et al.* 2008), suggesting the independence on MHC II expression of the exosomes' mother cell. In addition, functional failure of too low antigen-loads could have been the reason for the lacking T cell proliferation. However, *in vitro*, DCs are able to elicit strong antigen-specific T cell responses with small antigen amount (nanomolar and picomolar concentrations) (Banchereau and Steinman 1998; Mellman *et al.* 1998).

Exosomes can be internalized by two independent mechanisms. Either, they are taken up by the endocytic machinery or they directly fuse with the target cell's membrane, releasing its content into the cytoplasm (Thery 2011). The way of internalization strongly influences the shuttle of antigen into different compartments

and, consequently, the processing and presentation of antigen (Hilmenyuk *et al.* 2010). If keratinocyte exosomes are channeled into a different pathway than immune cell exosomes, it is conceivable that the differing effects are related to specific shuttle routes.

However, it is also possible that MPEK-derived exosomes did not fail to induce antigen-specific T cell responses but rather prevented T cells from proliferation as an anti-inflammatory mechanism. Along this line of speculation, keratinocyte-derived exosomes could exert protective functions, thus down-regulating excessive immune reactions. Indeed, serum-derived exosomes did prevent allergic sensitization in mice with allergic asthma (Almqvist *et al.* 2008). Interestingly, the immune-stimulating capacity of DC-derived exosomes significantly depends on the state of maturation. As mature DC-derived exosomes are considered the most potent immunostimulatory exosomes (Segura *et al.* 2005a; Zitvogel *et al.* 1998), immature DC-derived exosomes have immunosuppressive effects instead. The differential expression of co-stimulatory molecules is a possible explanation for this notion (Ruffner *et al.* 2009; Yin *et al.* 2013). However, with regards to their expression of co-stimulatory molecules, keratinocyte-derived exosomes are much more similar to immature DC-derived exosomes than to mature DC-derived ones (Chavez-Muñoz *et al.* 2009; Yin *et al.* 2013), suggesting an anti-inflammatory rather than a pro-inflammatory immunological role. *In vivo*, this finding could imply an anti-inflammatory effect of keratinocyte-derived exosomes for physiological and pathological immune processes.

5.3 Bidirectional entanglement of environmental conditions and exosomal composition

Analysis of exosomes generated under the influence of “atopic” or “psoriatic” cytokine cocktails highlighted the profound environmental regulation of exosomal composition that affected virtually all functional fields (Table 20 page 43). Compared to primary keratinocytes, the use of a cell line provided more stability and reproducibility of the prepared exosomes, thus achieving better conditions to

compare the proteomic composition. Given that exosomes contain a distinctive assembly of proteins depending on the cell type they originate from (Simpson *et al.* 2008), this specific fingerprint hints at the mother cell and/or its state of maturation, as demonstrated for dendritic cells and human keratinocytes (Chavez-Muñoz *et al.* 2009; Yin *et al.* 2013). Therefore, it is possible to generate different exosomes by interleukin treatment of the mother cell. Exosomes of "conventional" monocyte-derived DC (MDDC) and IL-4/IL-3-generated MDDC phenotypically reflected their MDDC of origin (Johansson *et al.* 2008). However, a selective enrichment of proteins prevents exosomes from being a miniature format of the cell itself (Ratajczak *et al.* 2006). In line, investigations of exosomes derived of primary cancer cells and metastatic cells discovered selective enrichment of certain proteins involved in metastasis and signaling in metastatic cell-derived exosomes (Ji *et al.* 2013). The specific protein composition of exosomes renders them as possible biomarkers. Especially in the fields of oncology and nephropathies, a lot of effort has been put on such biomarkers to improve early diagnosis and to predict the course of the disease (Dear *et al.* 2013; Simpson *et al.* 2009). Besides this diagnostic potential, exosomes may also provide therapeutic alternatives, as a variety of immunosuppressive exosomes for treating arthritis has been proposed (Yang and Robbins 2012). Moreover, IL-10 treatment of DC generated immunosuppressive exosomes, which were able to suppress inflammation and collagen-induced arthritis (Kim *et al.* 2005). Owing to their stability to phenotypic changes after injection, exosomes may qualify for therapeutic use (Zitvogel *et al.* 1998). Contrariwise, exosomes may also provide a therapeutic target. Circumstantial evidence suggests that extracellular vesicles derived from *Staphylococcus aureus* are involved in the pathogenesis of atopic dermatitis (Hong *et al.* 2010). These findings are in line with a proinflammatory role of epithelial cell-derivd exosomes and further emphasize a pathophysiological role of exosomes in various diseases (Kulshreshtha *et al.* 2013). Thus, diminution of these vesicles is predicted to ameliorate certain disorders. The mutual influence of exosomes and the environment emphasizes the possible entanglement of exosomes in (patho)-physiological processes and establishes a role for exosomes as potential therapeutic tools as well as targets.

5.4 Further considerations

Notwithstanding the demonstration of new functions of murine keratinocyte-derived exosomes, this study has raised further questions and has some obvious limitations:

a) The BMDC and T cells used were generated for every experiment from different mice. Although the experimental animals were raised under identical conditions and were used at the same age, such biological systems may show considerable inter-individual variations.

b) The experiments provided evidence for an immunomodulatory function of exosomes. Yet, it remains to be resolved whether keratinocyte-derived exosomes have a stimulatory or rather an immunosuppressive role in the cutaneous immune system and through which factors and pathways these effects are mediated. A feasible approach could be the demonstration of OVA in BMDC to exclude insufficient OVA transfer. Otherwise, it is not clear which quantity is needed to elicit an antigen-specific T cell response. Even if OVA would not have been visualized in BMDC, BMDC could still elicit a T cell response. Further, exosomes specifically influenced CD40 expression but not CD86, MHC I, or MHC II, thus strengthening the point of a specific response.

c) Due to the lack of comparable mouse models and syngeneic DC in human models, the experimental setup cannot be transferred easily to human keratinocytes. However, the results would be supported by the demonstration of similar effects when using different murine cell lines and primary keratinocytes.

6 Summary and Conclusion

Keratinocytes are able to influence cutaneous immunity by taking part in innate as well as in adaptive immune processes. To fulfill this task, the production of soluble factors has a key role. Exosomes, small membrane vesicles of endocytic origin and secreted by a variety of cells, may modify immune responses. However, little is known about the immunological function of keratinocyte-derived exosomes.

To address this question, the murine keratinocyte cell line MPEK was cultured *in vitro*, and the production of exosomes has been demonstrated. These exosomes were internalized by bone marrow-derived dendritic cells (BMDC), where they induced production of high amounts of IL-6, IL-10, and IL-12 as well as increased surface expression of CD40. When MPEK were incubated with antigen, they transferred it to their exosomes. However, antigen-loaded exosomes failed to induce an antigen-specific T cell response directly or via BMDC. When environmental conditions, mimicked by cytokine cocktails characteristic for two of the most common chronic inflammatory skin disorders, atopic dermatitis or psoriasis, were modulated, a profound impact on the molecular repertoire of exosomes was found. The specifically altered components comprised virtually all functional fields.

Even though MPEK-derived exosomes did not elicit an antigen-specific T cell response, they were clearly able to influence BMDC function and phenotype. Further, interleukins and IFNγ markedly influenced the molecular exosome composition. Together, these findings suggest an immunomodulatory function for MPEK-derived exosomes and a crucial effect of environmental conditions on exosome composition end presumably even function.

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8 Appendix

8.1 Supplemental data

Table 21: CD40 MFI

BMDC	medium	Exo	Exo IFNγ	Exo OVA	Exo OVA IFNγ	OVA
Exp: 1	634	-	1745	-	1627	775
Exp: 2	554	1071	-	1352	1393	610
Exp: 3	920	2962	2744	2558	2789	1103
Exp: 4	850	1840	1912	1296	1231	852
Exp: 5	478	1177	884	993	948	574

Table 22: CD86 MFI

BMDC	medium	Exo	Exo IFNγ	Exo OVA	Exo OVA IFNγ	OVA
Exp: 1	2136	-	4405	-	4116	2290
Exp: 2	1807	1803	-	2175	2207	1657
Exp: 3	1529	1755	1673	1462	1459	1486
Exp: 4	2705	2736	2981	2955	2373	2742
Exp: 5	2739	2078	2097	2101	2216	2457

Table 23: MHC I MFI

BMDC	medium	Exo	Exo IFNγ	Exo OVA	Exo OVA IFNγ	OVA
Exp: 1	347	-	401	-	364	352
Exp: 2	396	471	-	515	503	434
Exp: 3	655	769	667	708	567	674
Exp: 4	551	483	519	436	426	552
Exp: 5	305	329	309	295	361	322

Table 24: MHC II MFI

BMDC	medium	Exo	Exo IFNγ	Exo OVA	Exo OVA IFNγ	OVA
Exp: 1	1506	-	3319	-	3104	1684
Exp: 2	7524	3265	-	4380	3568	6637
Exp: 3	3525	3266	3064	2864	2242	4211
Exp: 4	3365	2392	2350	2203	2124	4282
Exp: 5	6384	3427	3585	3915	3823	6446

Table 25: IL-6 pg/ml

BMDC	medium	Exo	Exo IFNγ	Exo OVA	Exo OVA IFNγ	OVA
Exp: 1	94	-	196049	-	259927	388
Exp: 2	55	67627	-	150508	190331	261
Exp: 3	135	250644	305798	137684	262485	447
Exp: 4	297	1398414	1586590	1605211	1626537	695
Exp: 5	93	227987	204520	223765	270825	335

Table 26: IL-10 pg/ml

BMDC	medium	Exo	Exo IFNγ	Exo OVA	Exo OVA IFNγ	OVA
Exp: 1	0	-	1046	-	1013	0
Exp: 2	0	403	-	786	709	0
Exp: 3	0	154	249	64	153	0
Exp: 4	0	147	200	309	271	0
Exp: 5	0	158	114	149	141	0

Table 27: IL-12 pg/ml

BMDC	medium	Exo	Exo IFNγ	Exo OVA	Exo OVA IFNγ	OVA
Exp: 1	0	-	142	-	281	0
Exp: 2	0	42	-	121	308	0
Exp: 3	0	175	302	87	211	2
Exp: 4	0	124	148	161	161	0
Exp: 5	5	113	83	104	220	0

Table 28: Exosome production µg/µl

Exp	1	2	3	4	5	6	7	8	9	10
Exo	1,05	1,70	3,00	1,00	0,28	0,30	0,54	0,54	0,98	0,80
Exo IFNγ	0,70	1,90	2,50	1,30	0,27	0,34	0,28	0,60	0,71	0,63

8.2 Publications

8.2.1 Publications

Kotzerke K, Mempel M, Aung T, Wulf GG, Urlaub H, Wenzel D, Schön MP, Braun A (2013): Immunostimulatory activity of murine keratinocyte-derived exosomes. *Exp Dermatol* 10, 650-655

8.2.2 Presentations with published abstract

Oral presentation and poster presentation at the 42nd Annual ESDR Meeting in Venice and thus an abstract has been published:

Kotzerke K, Aung T, Wulf GG, Urlaub H, Wenzel D, Schön MP, Mempel M, Braun A (2012): Immunostimulatory effects of exosomes from murine kerationcytes. *J Invest Dermatol* 132, S1

Curriculum Vitae

Mein Name ist Kristina Kotzerke, ich wurde am 31.05.1988 in Hannover als Tochter von Kristin Kotzerke, geb. Trautschold, und Jörg Kotzerke, geboren.

In den Jahren 1994-98 besuchte ich die Grundschule Großburgwedel, um 1998 für zwei Jahre auf die Orientierungsstufe Burgwedel zu wechseln. Ab 2000 besuchte ich das Gymnasium Großburgwedel, an dem ich 2007 meine Hochschulreife erwarb (Abiturnote: 1,4). Das Schuljahr 2004-05 absolvierte ich in den USA an der Madison High School, South Dakota.

Im Oktober 2007 begann ich das Studium der Humanmedizin an der Georg-August-Universität Göttingen. Im September 2009 bestand ich den Ersten Abschnitt der Ärztlichen Prüfung (Note: 1,0).

Wissenschaftlich befasste ich mich seit 2010 mit „Immunomodulatory activity of murine keratinocyte-derived exosomes“ in der Klinik für Dermatologie, Venerologie und Allergologie der Universitätsmedizin Göttingen (Betreuer der Promotion: Prof. Mempel und Prof. Schön). Hierfür legte ich ein Forschungssemester ein, um mich ganz der experimentellen Arbeit widmen zu können. Die Ergebnisse konnte ich bereits auf einem internationalen Kongress vorstellen und hierzu auch ein Reisestipendium einwerben: Tagung der European Society for Dermatological Research in Venedig 2012. Des Weiteren wurden Teile dieser Arbeit bereits publiziert (Kotzerke K, Mempel M, Aung T, Wulf GG, Urlaub H, Wenzel D, Schön MP, Braun A (2013): Immunostimulatory activity of murine keratinocyte-derived exosomes. *Exp Dermatol* 10, 650-655).

Das Praktische Jahr absolviere ich vom Februar 2013 bis zum Januar 2014 in der Abteilung Innere Medizin des Ameos Klinikums Alfeld, in der Abteilung Chirurgie des Krankenhauses Dresden-Friedrichstadt und in der Abteilung Neurologie des St. Bernward Krankenhauses Hildesheim.

Im Frühjahr 2014 werde ich voraussichtlich das Studium mit dem Zweiten Teil der Ärztlichen Prüfung abschließen.

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