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In vivo Charakterisierung der MHC-Expression und endocytotischer Transportprozesse exogener Antigene in humanen Dünndarmenterozyten von Morbus Crohn Ileitis Patienten und Kontrollen

***In vivo* characterization of the MHC expression and the endocytic trafficking pathways of luminal antigens within intestinal epithelial cells in Crohn's ileitis patients and controls**

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Cells are Life. The rest is just detail.

(American Society for Cell Biology- ASCB)

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Abbreviations

Ag/Ags	antigen(s)
APC/APCs	antigen presenting cell(s)
APM	apical membrane
BSA	bovine serum albumin
C°	degrees centigrade
CD	Crohn's disease
Cr	crypt
Cyt	cytosol
DCs	dendritic cells
EE/EEs	early endosome(s)
GALT	gut associated lymphoid tissue
IBD	inflammatory bowel disease
ICS	intercellular space
IEC/IECs	intestinal epithelial cell(s)
IEM	immunoelectron microscopy
Ii	invariant chain
IL	interleukin
OVA	ovalbumin
LAMP-2	lysosome-associated membrane protein-2
LE(s)	late endosome(s)
LP	lamina propria
Lu	lumen
Lys	lysosomes
MHC	major histocompatibility complex
MIICs	MHC II-enriched compartments
Mit	mitochondria
MLL(s)	multilaminar lysosome(s)
MSE	multivesikuläre späte Endosome
MVB/MVBs	multilaminar body(s)
MVLE(s)	multivesicular late endosome(s)
Nuc	nuclei
TGF	transforming growth factor
UC	ulcerative colitis

Introduction

Anatomy and histophysiology of the intestinal mucosa

The main function of the gastrointestinal system (including the mucosa, as the main functional site) is to break apart food for absorbtion into the body. This natural process occurs by ingestion, fragmentation, digestion, absorbtion and elimination of waste components. The gastrointestinal tract is essentially a muscular tube lined by a complex mucosal membrane, both components having regional differences. The muscular component gives strength to the gastrointestinal wall as well as moving the food along it (peristalsis), while the mucous membrane has a protective (oral cavity, oesophagus and anal canal), secretory (stomach), absorbtive role (entire small intestine) or a combination of these in different parts of the tract, for example absorbtive/protective in the entire large intestine (1).

From histophysiological point of view, the gastrointestinal tract has four distinct functional layers: *mucosa*, *submucosa* (collagenous and adipose supporting tissue)*, muscularis propria* and *adventitia* (the other layer of supporting tissue).

The mucosa

The mucosa is made up of three components:

1. *epithelium,* a single layer of approx. 20 μm thickness, formed by intestinal epithelial cells (IEC, or enterocytes) (1),
2. *lamina propria* (LP), a loose supporting and vascularized tissue with diffuse population of so-called *lymphocytes* and plasma cells; lymphocytes are a class of white blood cells deriving from the Thymus, bearing variable cell-surface receptors for so-called *antigens; Ags* (Ag) are considered “non-self” for our body, mostly foreign molecules, which have the ability to generate an immunological response, respectively to elicit antibody production (1, 2) and
3. a thin smooth muscle layer, the *muscularis mucosae*, responsible for movement and folding of the mucosa. The muscularis mucosa separates the mucosa from the submucosa.

The mucosa of the small intestine is arranged into finger-like projections, called *villi,* which increases the absorbtive surface area, and intervening short glands reaching

into the LP, called *crypts* (Lieberkühn). The epithelium is constantly renewed by fresh cells arising from the 10–20 crypts of Lieberkühn surrounding each villous (1, 3).
The villi are covered at the luminal surface by a simple layer of thousands of tall columnar IECs. Beside IEC majority, the epithelium includes several other cell populations like: *goblet cells* (production of mucin, which is composed of glycosylated proteins that form a protective layer of gel-like mucus over the surface epithelium), *Paneth cells* (defensive function), *neuroendocrine cells* (for local acting and regulating hormones), *stem cells* (primal cells which have the ability to renew themselves and also to differentiate into a diverse spectrum of specialized cell types, localized also at the base of the crypts) and *intraepithelial lymphocytes* (mostly T cells). Each of these cells contributes in a unique way to mucosal defense and the maintenance of barrier integrity (1).

Intestinal epithelial cells (IECs), the most abundant cell type in the gut, are the main absorptive and digestive cells of the bowel epithelium and the focus of the present study. The average turnover of IECs is between 24-96 hours in the small intestine (4). IECs are polarized epithelial cells, which have an apical (luminal), a basolateral and a basal surface. As a general feature of polarized cells, the basolateral membrane contacts neighboring cells and the underlying tissue, whereas the apical membrane faces the lumen of an internal organ. The apical surface of IECs exposes to the lumen up to 3000 uniform *microvilli* (approx. 1µm in length) forming the *brush border* of IECs (1).
The membrane domains (apical and basolateral), are separated by *tight junctions* or *zonula occludens* (specific anatomical structures, found only in vertebrates, comprising closely associated areas of two cells whose membranes join together forming a barrier to fluids). Tight junctions help to prevent mixing of apical and basolateral membrane components and seal the epithelium (1, 5).
It is textbook knowledge that IECs, as the first line of cells towards the lumen, provide a crucial mechanical barrier function, although being only a single cell layer approx. 20 µm thin. IEC's membranes, as well as the tight junctions, represent an important physical barrier preventing the penetration of potential harmful microorganism. Beyond the apparent passive barrier, IEC provide the classical nutrient and ion transport function. As a major point of contact for enormous amounts of enteric Ags (food Ags, viruses, bacteria, parasites or the by-products of these organisms), IECs

also assume an active role in mucosal immunity as nonprofessional Ag presenting cells, by several mechanisms (see chapter “Antigen presentation in intestinal epithelial cells”).

IECs defend epithelial surfaces by actively secreting a variety of antimicrobial proteins (6). Furthermore, IECs possess extensive cellular contacts with several distinct populations of T lymphocytes both within the epithelium (*intestinal epithelial lymphocytes*) and in the underlying lamina propria (*lamina propria lymphocytes*). There have been surprisingly many molecules identified, which mediate the physical interaction between T cells and IECs (7). The complete functional consequences of these interactions are not well understood yet. IECs are able to release a wide variety of *cytokines* (special proteins produced by some immune cells, that influence the behavior of other cells), both constitutively and after invasion with bacterial pathogens (8).

Basically, because of the continuous contact with the external environment, as a portal of entry of pathogenic organisms, the mucosa owns multiple defense mechanisms including the *gut associated lymphoid tissue* (GALT) (1, 9).

The GALT*,* as part of the mucosal associated immune system is found in all parts of the gastrointestinal tract except the stomach. It represents the largest organized lymphoid system of the human body and has the difficult task of virtuous orchestration of the complex mechanisms of mucosal immunity. The GALT is constantly exposed to a variety of antigenic constituents like dietary Ags, commensal bacteria and different germs. From anatomical point of view, the GALT comprise the *Peyer’s patches* (especially in the small intestine), *mesenterial lymph nodes* and the *intraepithelial lymphocytes*, a large number of lymphoid cells scattered throughout the lamina propria. The largest aggregates are the *Peyer’s patches* of the small intestine formed by groups of lymphoid follicles located in the mucosa where they bulge luminally in a dome-like feature. The follicle is similar to lymph nodes, consisting of a germinal centre (proliferating and maturing B cells) and the overlying epithelium, formed by follicle-associated low cuboidal epithelial cells, or *microfold cells* (M-cells), specialized for transcytosis (meaning a transcellular cargo) and Ag uptake from the gut lumen into underlying Peyer’s patches (9).

The GALT uses a large arsenal to protect the host against aggressive immune responses to luminal constituents, including a strong physical barrier, luminal enzymes (that can alter the Ags), regulatory T cells, as well as the production of a

specific antibody- the secretory immunoglobulin A for the hostile gut environment (10-12).

Antigen uptake, processing, and presentation

It is textbook knowledge that in order to exist, every cells must perform several fundamental activities (as a part of a multicellular “community”), including the uptake of essential nutrients, the regulated interaction with the external world, the ability to mount an effective defense against invading microorganisms, as well as the transmission of neuronal, metabolic and proliferative signals (1).

Endocytosis

Cells are able to take up various molecules and particles from the surrounding medium by a distinct process termed *endocytosis.* In endocytosis, the material to be internalized is surrounded by an area of plasma membrane, which then buds off inside the cell, as a selective inclusion in order to form a vesicle containing the ingested material. The term “endocytosis” was introduced by Christian De Duve in 1963 to include both the ingestion of large particles (such as bacteria) called phagocytosis (cell eating) and the uptake of fluids or macromolecules in small vesicles known as pinocytosis (cell drinking) (13).

In polarized cells (e.g., epithelial cells), internalized receptors or molecules can also be transferred across the cell to the opposite domain of the plasma membrane, a process called *transcytosis*. There are of course trafficking processes out of the cell, *exocytosis,* and between separate cellular compartments.

Endocytosis serves to maintain a homeostasis (cellular balance) by recovering protein and lipid components inserted into the plasma membrane by ongoing secretory activity. Cargo transport can occur trough several mechanisms, such as *passive diffusion* (dependent on the concentration gradient across the plasma membrane), *facilitated diffusion* (also a passive concentration-dependent mechanism requiring a protein carrier molecule), *active transport* (independent of the concentration gradient, requiring energy), *bulk transport* (molecule cargo into, out of, or between cellular compartments, mediated by subcellular structure, like *coated vesicles;* there are specific *endocytosis-, exocytosis-* and *intracellular transport*

vesicles) and *transmembrane signaling* (implies the binding of a signaling molecule to a membrane receptor, usually enzyme, leading to the activation of the latter) (1, 14).

Cells are elaborately subdivided into functionally distinct, membrane-enclosed subcellular compartments. Each compartment, or organelle, contains its own characteristic set of specialized catalyzing proteins called *enzymes* and other specialized molecules, and complex intercompartmental transport systems. Proteins confer each compartment its characteristic structural and functional properties. They accelerate the reactions that occur in each organelle and selectively transport small molecules into and out of its interior, or lumen. Proteins also serve as organelle-specific surface markers that direct new deliveries of proteins and lipids (fats) to the appropriate organelle (15).

Organells of the endocytic system

The importance of the endocytic system relies in providing the antigenic import and generation of antigenic peptides followed by the association to specialized molecules (see chapter “Antigen presentation”). These events are fundamental because only processed and loaded antigenic material can activate potent effector cells (T cells) resulting in the specific immune response.

As observed about one hundred years ago by the scientist Elie Metchnikoff, specific material taken up by endocytosis was degraded after encountering an acidic internal environment, representing the endosomal system (14).

During the past decade, the basic organization of the endocytic pathway was elucidated, particularly in the case of the internalization of protein ligands bound to cell surface receptors (14). By “dissecting” the endocytic vesicular transport system, four classes of endocytic organelles (without counting the intermediary organelles) are typically distinguished based on their relative kinetics of labeling by endocytic tracers: early endosomes, late endosomes, recycling vesicles, and lysosomes (Lys) (16). Protein trafficking from the cell surface to Lys involves distinct steps of protein sorting and occurs via different types of vesicular intermediates. The precise relationship among these structures has to be determined, and, in fact, may not exist because of the great plasticity and dynamics of the system. It is also almost impossible to recognize these structures on the basis of morphology or position in the cytoplasm alone (14). As described earlier, endocytosis is typically started by the

formation of clathrin-coated vesicles at the plasma membrane, which bud from the plasma membrane as a selective inclusion, resulting in the delivery/fusion of receptor-ligand complexes to specific organelles, called *early endosomes* (EEs).

Early endosomes (EEs) are the first and in the same time the major sorting compartment of the endocytic pathway in the peripheral cytoplasm, also known as pre-endosomal compartments. Here, receptor-ligand complexes dissociate, thus resulting in the return of free receptors to the plasma membrane in recycling vesicles. Actually, it has become clearer in the past years that endosomes mediate even more sophisticated sorting events, playing an important role in the targeting of membrane proteins to their correct plasma membrane domains in polarized epithelial cells, to a variety of specialized endosome-derived recycling or secretory vesicles, and to the *trans Golgi network* (TGN, organelle responsible for processing and packing macromolecules like proteins and lipids that are synthesised by the cell) (14).
In addition, the EEs of polarized cells can transfer endocytosed proteins between different domains of the plasma membrane, for example, between the apical and basolateral domains of epithelial cells (17). Selective protein transport from the plasma membrane towards EEs is mediated by small (<100 nm in diameter) and short-lived (approx. 1 min) vesicles coated with clathrin (18). The majority of internalized molecules are recycled back to the cell surface, whereas a small part is selectively incorporated in transport intermediates, having the late endosomes as destination (14, 19). These intermediates, named *endosomal carrier vesicles*, differ evidently from the EEs, being relatively large (approx. 0.5 μm) and long-lived (approx. 15-30 min) (19-21). Finally, the EEs gradually mature into late endosomes (LEs), which are the precursors to Lys (17).
One of the important changes during endosome maturation is the lowering of the internal pH from about 6.0-6.2 in EEs to about 5.5 in LEs, as the result of the action of a membrane H^+ pump. This acidic pH leads to the dissociation of many ligands from their receptors within EEs. Following this uncoupling, the receptors and their ligands can be transported to different intracellular destinations. The pH also plays a key role in the delivery of lysosomal acid hydrolases from the TGN in the endocytic system (22, 23).
Ligands and membrane proteins destined for degradation in Lys are transported from EEs to LEs, which are located near the nucleus. The transport from early to late

endosomes is mediated by the movement of large endocytic carrier vesicles along microtubules. The LEs are more acidic than EEs (pH about 5.5 to 6.0) and are able to fuse with transport vesicles carrying lysosomal hydrolases from the Golgi apparatus, as discussed earlier.

LEs then mature into Lys as they acquire a full complement of lysosomal enzymes and become even more acidic (with pH of about 5). Within Lys, the endocytosed materials (both of endogenous and exogenous origin) are degraded by the action of acid hydrolases (17, 24). For example, a receptor endocytosed from the basolateral domain of the plasma membrane can be sorted in EEs for transport to the apical membrane. In some cells, this is an important mechanism for sorting membrane proteins (17, 22, 23).

It can be generally affirmed that the EEs serve as a sorting compartment, from which molecules taken up by endocytosis are either recycled to the plasma membrane or transported to LEs and Lys for degradation (14).

Endosomes are basically connected also to the biosynthetic pathway through selective trafficking pathways. Vesicles coated with clathrin associated with the so-called AP1 adapter complex mediate the delivery of newly synthesized lysosomal enzymes and lysosomal proteins from the TGN to endosomes, as well as perhaps AP3-coated vesicles (25). Protein recycling back to the TGN occurs from LEs (26), and perhaps from EEs (27).

Lysosomes (Lys) are the latest components of the endocytic pathway, the terminal degradation compartment (16). Morphologically, they are approx. 0,5 µm small membrane-enclosed organelles. In their simplest form, Lys are visualized as spherical electrone-dense vacuoles (28).

They contain the highest enzymatic concentration among all endocytic compartments meaning the lowest pH (approx. 5). The broad array of enzymes includes about 50 different degradative enzymes, capable of breaking down all types of biological structures (such as proteins, nucleic acids, carbohydrates, and lipids) by hydrolysation. Lys function as the digestive system of the cell, serving both to degrade material taken up from outside the cell and to digest obsolete components of the cell itself-in autophagy (29) or crinophagy (30). Lys label LAMP, an acronym for *lysosome-associated membrane protein* (CD107a and b), a specific marker protein (31). Variations in size and shape are a result of the different materials that have

been taken up for digestion. Thus, Lys represent morphologically diverse organelles defined by the common function of degrading intracellular material (16).

Beside the classical round and homogenous electron-dense aspect, there are also tubular Lys, and a multilamellar-featured Lys, also called l*ysosomal MIIC/multilamellar lysosome* (32, 33). Multilamellar Lys (MLLs) are lysosomal organelles found frequently in Ag presenting cells (APCs) of the immune system. They function also as major histocompatibility compartments (MHC) class II- peptide loading compartments, beside the classical MHC II-enriched compartment (MIIC, described in detail in the next chapter). MLLs measure from approx. 200 to 1000 nm in diameter and are morphologically characterized by an accumulation of internal membrane sheets (33).

The MLL ultrastructure and biogenesis is, however, still incompletely defined. By using high-resolution electron tomography on cryo-immobilized cells and subsequent 3D reconstructions of MLLs, Murk and colleagues (33) were able to describe in B-lymphocytes and dendritic cells (DCs) that the inner sheets are organized in concentric spheres without interconnections or connection with the outer lysosomal membrane. This membrane organization may suggest that MHCII molecules on the inner membranes are trapped within the volume enclosed by the outer membrane, and that only MHCII on the outer membrane is able to relocate to the cell surface. Based on the 3D membrane organization, Murk and colaborators proposed a model for MLL biogenesis, as follows: the complex internal membrane organization is characterized by the fact that there are no connections between the internal membranes and outer membrane. The internal membranes are independent membrane vacuoles and sheets, suggesting that they are trapped within the outer membrane. Therefore, MHCII molecules on the inner membranes are trapped inside the MLLs and cannot actively participate in T-cell activation at the cell surface (32, 33).

This spatial entrapement of MHC molecules leaves the MIICs as the major peptide loading and presenting organell.

As described for classical multivesicular LEs, MLLs can also form tubules that contain MHCII molecules. Tubule formation of MVBs is accomplished by back fusion of the inner membrane vesicles with the outer membrane, and leading to the loss of the inner membrane upon DC maturation (32, 33). Moreover, MLLs frequently show tubular extensions that project into the cytosol. Because these contain MHC II, there

has been proposed that the tubules are the potential site of MHCII exit (34). It remained still unclear whether the MHCII pool on the luminal membranes can contribute to MHCII expression on the cell surface.
It was proven that the activation of human monocyte-derived DCs leads to disappearance of most of the internal MHCII molecules from the MLLs and they shrink in size. They also display a typical organization of densely packed lipids that seem to lack the structure of membranes (33, 34).
Lys are also responsible for autophagy, the gradual turnover of the cell's own components (16).

Late endosomes (LEs) and multivesicular MHC II-enriched compartments (MIICs)

Late endosomal and lysosomal compartments have three major characteristics: they posses an acidic pH, have degradative enzymes and additionally express typical proteins from the LAMP protein family (31).
From electron microscopical view, LEs have a more spherical shape than EEs and are located often juxtanuclearly, being concentrated near the microtubule organizing center. In contrast to EEs, they have the appearance of multivesicular bodies (MVBs) (35). MIICs are ubiquitously present in all mammalian cells (35).
The dispute whether the origin and endosomal evolution between EEs and LEs is explained by vesicular transport or by the maturation of EEs still goes on. Since both models provide an intermediate between early and late endosomes, the difference lies in whether the intermediate is a specifc transport vesicle budded from the EE or is what remains after removal of certain components from an EE (19, 21, 36).
It has been described that endosomal/lysosomal compartments perform also other functions beside the classical protein degradation. In professional APCs, but also in several others, like epithelial cells, multivesicular LEs/MVB and Lys are enriched in MHC II molecules and are collectively called *MHC II-enriched compartments* (MIICs). MHC II molecules, also known as hystocompatibility Ags, are key molecules in the adaptive immunity, because they bind and present antigenic material, which is a pivotal step in the initiation of T cell immunity. Ag-specific stimulation of T cells ($CD4^+$ and $CD8^+$) requires the interaction of the *T cell receptor* with the MHC : Ag complexe (37, 38). Presentation of exogenous Ags has classically been attributed to MHC II

molecules and $CD4^+$ T cells. In general, MHC II molecules bind peptides formed in endocytic organelles (details in the chapter “Antigen presentation”).

MIICs have deep immune implications because they are the primary subcellular site of Ag processing, subsequent MHC II/peptide complex formation and Ag presentation (39, 40). MIICs are crucial subcellular compartments for MHC II molecule accumulation and regulation of Ag presentation in professional APCs, like DCs (32). In MIICs, MHC II molecules are loaded probabely with peptides that are derived from endocytosed Ags for presentation to helper T lymphocytes (30, 38, 40, 41).

Depending on their maturation phase, MIICs can have different morphologies, like multivesicular, multilamelar or both (42-44). Several studies on DCs have indicated that the MIIC structure is very dynamic and undergoes major shape changes during cell maturation (33, 34). The size of MIICs ranges between 300-500 nm in diameter and the morphologic composition is made up of a limiting membrane that encloses several small internal vesicles measuring approx. 40-90 nm in diameter (32, 45). The internal vesicles are formed by invaginations of the limiting membran budding into the lumen of the MVB (46, 47). In the same time, the inner vesicles haven been proved of not being connected to the limiting membrane (48). It is known that, for instance in immature DCs the free internal vesicles of MIICs function as a storage site for MHC II molecules (32).

Upon cell activation the internal vesicles of MVBs disappear, and the organelle accumulates MHC II on their external membrane, acquiring a tubular structure. The inhanced MHC II transport to the cell surface that occurs upon DC activation is most likely accomplished by back fusion of the internal vesicles with the endosomal outer membrane, releasing probably the MHC II content for transport to the cell surface (32, 48).

There have been proposed in B cells also subtypes of MIICs, like “early MIICs”, which expressed abundant MHC I and had only few vesicles, as the precursors of the classical MIIC, and probably the entry site into MIICs of newly synthesized MHC molecules (44).

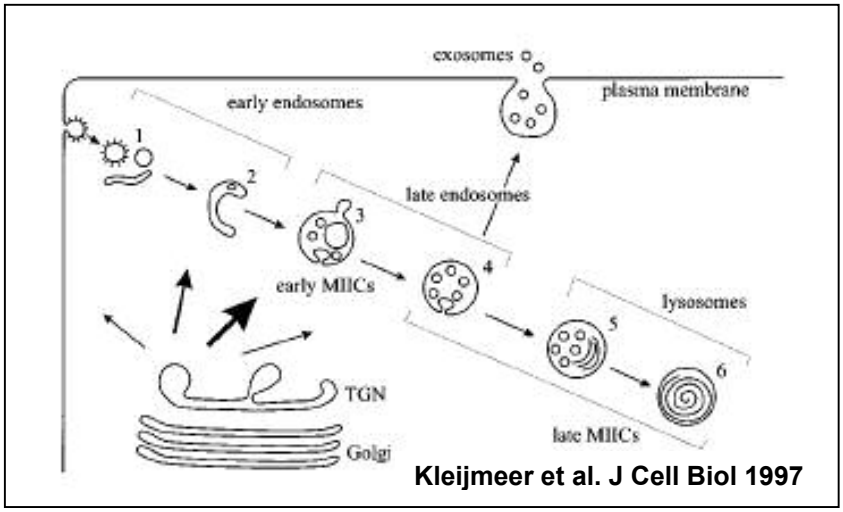
Another function of these internal vesicles is to degrade with their own hydrolases their contents, meaning a wide variety of protein and lipid substrates.

The exact vesiculation process that results in the formation of multivesicular endosomes/MIICs and the exisiting retrograde fusion consisting of backward fusion

of the vesicles into the endosomal limiting membrane are not fully understood yet. It was described by Kleijmeer and colleagues that lypopolysaccharide stimulation can lead to retrograde fusion events and hereby peptide-MHC II complexes reach the external endosomal compartment fulfilling the necessary “delivery mechanism” to the MIIC surface (32).

Bear MHC II molecules are distributed both on the internal vesicles and the external limiting MIIC membrane. Else than MHC II, MIICs of APCs (such as B lymphocytes) accumulate also other components required for Ag processing and presentation, including the invarinat chain (Ii), that targets MHC II from the Golgi to endocytic organelles. H-2M helps peptides to gain access to MHC II, and the proteases (especially cathepsin S) for the proteolysis (49, 50).

Beside the classical peptide loading, MIICs have another important function: the fusion with the plasma membrane of the cell and the release of internal vesicles in the extracellular environment, giving birth to *exosomes* (Ag-presenting vesicles) (51). In general, the fusion of MVBs with the plasma membrane could also be regarded as a transfer mechanism of MHC II/peptide complexes to the cell surface. These transfer processes could move MHC II from the limiting membrane of MVBs. In the same time, the majority of the MHC II in MVBs was described on the internal vesicles (51, 52). MIICs seem to play an important role also in the MHC I-mediated presentation of exogenous Ags.



Scheme 1. ***The different types of classical endocytic compartments and MHC II trafficking in professional Ag-presenting cells*** (from the observation made in B cells by

(43). MHC II:Ii complexes deriving from the trans Golgi network (TGN) are transported to early MIICs and presumabely in a reduced amount also to the plasma membrane and other endocytic compartments (marked by the arrows emerging from the TGN). In the endocytic pathway, MHC II/Ii complexes moove forward in the endocytic route parallel with the endocytosed Ags, both being increasingly exposed to crescent proteolytic activity (and decreasing pH). This results in the degadation of Ags into peptides and on the other hand into the cleavage of Ii, (probably beginning with early MIICs/type 3 and progressing in later compartments). After deblocking MHC II from the Ii-chain peptide fragment (CLIP fragment), the antigenic peptides are free to bind to the MHC II molecules. This complexation (peptide loading process) is catalyzed by HLA-DM. Peptide loading of class II starts in early MIICs and proceeds in later compartments, possibly depending on the type of Ag and MHC II. The next important step is the exoscytosis with deposition of the MHC:peptide complexes at the cell surface, by which the limiting membrane of MIICs is incorporated in the plasma membrane. During this process, the internal vesicles of MIICs are secreted/externalized as exosomes. By exocytosis, the MHC/peptide escape of acidic degradation in lysosomes (Lys). It is of course to expect the existence of other pathways of MHC II/peptide transport to the cell surface that remain to be defined.

Antigen presenting cells` derived exosomes

Exosomes are a very interesting and specific population of secreted small vesicles, (approx. 30-90 nm in diameter). The origin resides in the exocytic release of the internal vesicles of the MIICs, being considered as Ag presenting vesicles.

The first description of their endocytic origin goes back to 1983. In cultured reticulocytes, small vesicles were present inside large multivesicular endosomes, contained transferrin receptors, a marker used to follow endocytosis and the recycling of cell-surface proteins (being internalized from the plasma membrane) (53).

These microvesicles seemed to be formed by invagination and budding from the limiting membrane of LEs, resulting in vesicles that contained cytosol and that exposed the extracellular domain of transferrin receptors at their surface. There have been observed by electron microscopical analysis clear fusion profiles of multivesicular endosomes with the plasma membrane in several cell populations, leading to the secretion of the internal vesicles into the extracellular environment. Analysis of the protein composition of exosomes revealed insight into the biogenesis of exosomes. Interestingly, all of the exosomal proteins that have been identified are found in the cytosol, in the membrane of endocytic compartments or at the plasma membrane. It has been recently published that surprisingly mast cell exosomes contain nuclei acids, like RNA and mRNA, suggesting the exosomal role in intercellular signaling as genetical messenger (54).

From the present knowledge, exosomes do not contain any proteins of mitochondrial, endoplasmic reticulum or Golgi apparatus origin. Exosomes contain proteins that are involved in specific cell functions, e.g. exosomes are abundant in MHC II molecules in all cells that do express MHC II. Exosomes from DCs also contain CD86, which is an important costimulatory molecule for T cells. The most abundant protein families that were found in exosomes are the tetraspanins, including CD9, CD63, CD81 and CD82, which are enriched in exosomes from virtually any cell type. Tetraspanins interact with many protein partners, including MHC molecules and integrins. These aspects indicate their involvement in the organization of large molecular complexes and membrane subdomains.

Exosomes hold also a series of cell-specific transmembrane proteins, including:

1. integrins,
2. immunoglobulin-family members, such as intercellular adhesion molecule 1 (ICAM1=CD54) on B cells, A33 Ag on IECs and P-selectin on platelets or
3. cell surface peptidases, like dipeptidylpeptidase IV (CD26) on IECs and aminopeptidase N (CD13) on mastocytes.

Various metabolic enzymes (such as peroxidases, pyruvate and lipid kinases, and enolase-1) are found in exosomes from IECs and human DCs. Still, the mechanisms for sorting proteins into the internal vesicles of multivesicular compartments and, hence, to exosomes are poorly understood. Very little is known about the molecular machinery that is involved in the fusion of multivesicular compartments or secretory Lys with the plasma membrane (55).

The majority of the studies that analyzed the exosomal function used APCs-derived exosomes. In a pioneering study, Raposo *et al.* showed that exosomes that are secreted by EBV-transformed B cells stimulated human $CD4^+$ T cell clones in an Ag-specific manner (51). T-cell stimulation by exosomes produced by rat mast cells that were engineered to express mouse or human MHC class II molecules has also been reported recently (56). Beyond this, Zitvogel and colleagues showed in 1998 that exosomes produced by mouse DCs and pulsed with tumour peptides induce the rejection of established tumours, meaning an *in vivo* anti-tumoural immune response (57). It can be speculated, therefore, that exosomes have the potential to be used as vectors for vaccination in cancer immunotherapy. Indeed, DC-derived exosomes express high levels of functional MHC class I– and class II–peptide complexes,

together with CD86 molecules, and theoretically might substitute for DCs to elicit MHC class I- and class II-restricted T cell responses and tumour rejection (55).

Intestinal epithelial cells` derived exosomes

Basically, every cell that contains multivesicular endocytic compartments could potentially secrete exosomes. Also IECs from human IEC lines HT29 and T84 have been described to set exosome-like vesicles free from both the apical and basolateral sides. This release was significantly increased by the presence of interferon gamma (IFNγ) (58). Recently evidence has been brought that exosomes derived from the murine IEC line MODE K display MHC I and MHC II molecules (upregulated by IFN-γ) and tetraspan proteins like CD9, CD81, CD82, and that they are all potentially implicated in binding to target cells (59). Still, *in vivo* evidence for human IECs' derived exosomes are lacking.

Antigen processing

Ag processing is a biological process that prepares Ags for presentation to special cells of the immune system (e.g. T lymphocytes). This process involves two distinct pathways for processing of Ags from an organism's own proteins or intracellular pathogens (e.g. viruses), or from phagocytosed pathogens (e.g. bacteria). The subsequent presentation of these Ags on MHC I or MHC II molecules depends on which pathway is used. Both MHC I and MHC II are required to bind Ag before they are expressed on a cell surface. The endogenous pathway is used to present cellular peptide fragments on the cell surface on MHC I molecules. For this purpose, proteasomes (large barrel-like enzymatic complexes located in the cytoplasm) break the protein up into peptides consisting of approx. nine amino acids, suitable for fitting into the peptide-binding groove of MHC class I molecules. Once the peptide is transported into the lumen of the endoplasmic reticulum (ER, a cellular organelle, more precise a network of vesicles and tubules with multiple function like protein folding, secretion, transport, etc.) it binds to the cleft of the expectant MHC class I molecule, stabilizing the MHC and allowing it to be transported to the cell surface by the Golgi apparatus. The exogenous pathway is utilized by professional APCs to present peptides derived from proteins that the cell has endocytosed. Endocytosed proteins are degraded by acid-dependent proteases in endosomes (as described in

the chapter “Endocytotic system”). The peptides are presented on MHC class II molecules (60-62).

Antigen presentation in professional antigen presenting cells

The basis of adaptive immunity lies in the capacity of immune cells to distinguish between the body's own cells and infectious pathogens. Ag presentation is the specific immune process in which specialized APCs capture Ags and then enable their recognition by T cells. The activation of T cells by APCs leads to their proliferation and differentiation into “armed” effector T cells. Effector T cells can mediate a variety of functions, among the most important one is the killing of infected cells by $CD8^+$ cytotoxic T cells and the activation of macrophages by T_H1 cells, which together assemble the cell-mediated immunity. The activation of B cells by both T_H2 and T_H1 cells produces different classes of antibodies, thus establishing the humoral immune response (2).

By far, the most distinctive feature of APCs relies in the expression of costimulatory molecules, of which the B7.1 and B7.2 molecules are the best characterized. The three cell types that serve as prototypical APCs are *DCs, macrophages,* and *B cells,* each having a distinct function in eliciting the immune responses. DCs are considered to be the most potent activators of naïve T cells. Tissue DCs take up Ags by phagocytosis and macropinocytosis and are stimulated by infection to migrate to local lymphoid tissue, where they differentiate into mature DCs expressing costimulatory molecules. The macrophages efficiently ingest particulate Ags (e.g bacteria) and are induced by infectious agents to express MHC II molecules and costimulatory activity.

As described in the previous chapter, in professional APCs, processing of internalized Ags and subsequent loading onto MHC II is ascribed especially to MIICs (32, 43, 63). The unique ability of B cells to bind and internalize soluble proteic Ags via their receptors is important in activating T cells to this class of Ag, additionally to the fact that costimulatory molecules are also induced on the B cell. In all three types of APCs, the expression of costimulatory molecules is activated in response to signals from receptors that also function in innate immunity by announcing the presence of infectious agents (2).

There are several cells that act as APCs in the gastrointestinal mucosa. Among them, the DCs are considered to be the most efficient and serve as prototypical

APCs (64). DCs have a pivotal role in inflammatory conditions like IBD, being able to acquire different Ags and transport them to lymphoid tissue where they have the unique ability to activate naïve T cells (65). Additionally to the Peyer's patches and lymph nodes, DCs are found in large numbers in the LP, from where they extend their dendrites in luminal direction, through the tight junctions between IECs (66) and presumably in the epithelium (67). This close positioning to the gut lumen makes a direct Ag sampling possible (66, 68).

Classical MHC-dependent Ag presentation occurs in two main pathways.

1. The exogenous Ags are engulfed in coated pits being delivered to the endocytic pathways, where they are processed and bound to the Ag-binding groove of the complementary MHC II molecules. The Ag binding occurs through the displacement of the Ii, which stabilizes the MHC molecule, resulting in an MHC II-Ag complex. The Ii displacement process is facilitated by HLA-DM. MHC II-Ag complex is translocated from endosomal compartments to the external membrane in order to be displayed to the T-cell receptor of $CD4^+$T cells (43, 69-72).

2. Endogenous peptide Ags are processed in large proteasomes of DCs and trafficked by transport associated proteins (TAP-1 and TAP-2) from the cytosol into the endoplasmic reticulum (61, 73).

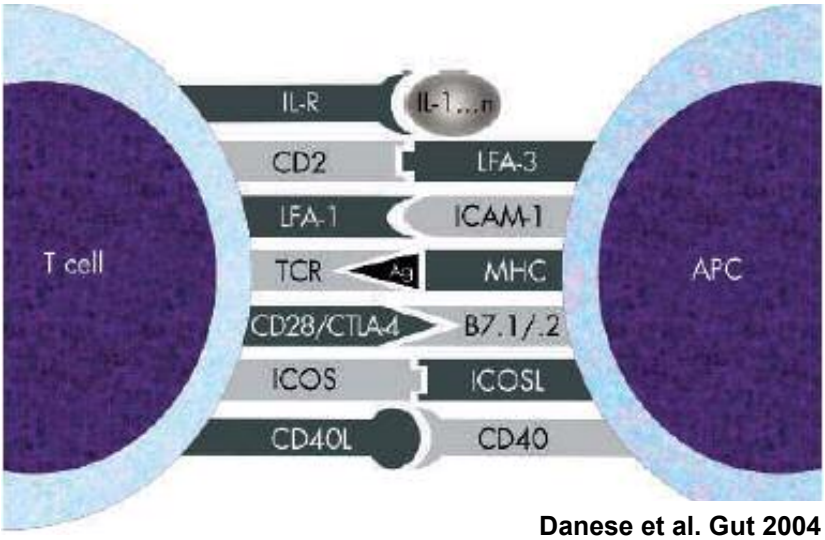
Here, they combine with MHC I molecules and β-2 microglobulin and are subsequently transported to the membrane surface for $CD8^+$ T cells recognition (61).

Ag-loaded DCs can migrate from the LP to the draining lymph nodes, where they present Ags to naïve T cells (74). Additional trafficking pathways are via microfold cells (M cells) of the follicular associated lymphoid tissue in the small intestine (75).

DCs can also engolf apoptotic epithelial cells (76) or can take up IEC-derived exosomes (58, 77). It is presumed that an altered immunoregulation, or an ineffective antibacterial activity, as well as genetically determined defective mucosal barrier function, can result in a persistent pathogenic immune response leading to a chronic gastrointestinal inflammation in susceptible individuals (12).

Presentation of exogenous Ags has classically been attributed to MHC II molecules and $CD4^+$ T cells. However, recent data indicated the presentation of exogenous Ags to $CD8^+$ T cells via MHC I, by the "cross-presentation" phenomenon (37).

There are evidence of cross-presentation of soluble Ags like OVA by professional APCs, if they are injected in a cell-associated form or by simple cell adsorbtion (78) (further details in the chapter *Cross-presentation*).



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Scheme 2. ***from (79) Breef representation of all the major molecular receptor-ligand pairs implicated in APC:T cell interaction***, meaning: activation, downregulation, co-stimulation, and none the less amplification of immune responses. (Ag, antigen; APC, antigen presenting cell; CTLA, cytolytic T lymphocyte associated Ag; ICAM, intercellular adhesion molecule; ICOS, inducible costimulator; IL-1…n, interleukin 1 to n; IL-R, interleukin receptor; LFA, lymphocyte function associated antigen; MHC, major histocompatibility complex; TCR, T cell receptor).

Antigen presentation in intestinal epithelial cells

The gut epithelium has traditionally been regarded as an absorptive tissue and a physical barrier positioned between the environment and the host. Numerous studies provide increasing evidence for an active role of IECs in sampling luminal Ags and modulating related immune responses, as non-professional APCs (80).

Bland and colleagues described in 1986 for the first time that IECs can function as APCs to $CD4^+$ T cells in rats (80-82), followed by the evidence in murine IECs (83) and human IEC lines (84). Several research groups had documented that normal IECs can express constitutively MHC II molecules on both the luminal and basolateral surface. Furthermore, IECs have the capacity to take up soluble Ags *in vivo* and *in vitro* and process Ag appropriately, and, subsequently, to present them to class II–restricted T cells, as non-professional APCs (IECs in human, rat and mouse) (80-83, 85-88). Along with the constitutive expression of MHC II molecules, IECs *do* also express molecules necessary for functional Ag loading onto MHC II, e.g. Ii and HLA-DM, and MHC I molecules (87). The constitutive expression of MHC II in IECs is

up-regulated during mucosal inflammation in the course of Crohn's disease (CD) (89).

The stimulation of cultured IECs with IFNγ results in the expression of MHC class II molecules, and gives the capacity to present Ags to a human TcR-transfected murine T cell hybridoma or T cell lines (84, 90).

Ag presentation by IECs under constitutive non-inflammatory conditions is suggested to result in $CD4^+$ T cell anergy (a state of none-responsiveness to certain immunogenic components/Ags), or induction of regulatory T cells ($CD4^+$ and $CD8^+$ T cells). It is well known that inappropriately activated $CD4^+$ and $CD8^+$ T cells proliferate and sustain mucosal inflammation, e.g. in inflammatory bowel disease by secretion of various pro-inflammatory cytokines (91-93). On the other hand, Ag presentation by IECs during CD inflammation was shown to stimulate pro-inflammatory $CD4^+$ and $CD8^+$ T cells (80, 90, 94-96).

Like in professional APCs, MHC II-restricted presentation of Ags by IEC lines was suggested to involve acidic endosomal compartments and the activity of acidic hydrolases, features of specialized LEs (84, 97).

Several groups, including ours, described the uptake and intracellularly sorting of exogenous/luminal Ags into LE/MIICs of IECs both *in vitro* and *in vivo*. The internalization and intracellular trafficking of exogenous Ags such as ovalbumin (OVA) into MIICs has been described in detail in jejunal murin IECs (93, 98). The translocation of another exogenous Ag towards LEs and co-localization with MHC II in the same compartment was demonstrated in human jejunal IECs, both in healthy and Coeliac disease patients (99). Furthermore, our group was able to describe *in vivo* the subcellular transport of endoscopically administetered OVA towards MIICs of human colonic epithelial cells, under basal and inflammatory circumstances (in healthy and CD patients, respectively) (100). Altogether, these data strongly suggest an important function of MIICs in MHC II-related Ag presentation by IECs, comparable to professional APCs. In light of the Ag presenting and pro-inflammatory T cell stimulation capacity of IECs, the elucidation of the Ag trafficking and mechanisms of Ag presentation via MHC II by ileal IECs, especially the associated endocytic pathways of exogenous Ags, is of crucial importancy because the bowel inflammation in CD characteristically affects also the terminal ileum. The endocytic processes within ileal IECs in healthy controls and CD patients is of high interest to the scientific community and represents one of the main focuses of this study.

Additional features in favor of the APC qualities of IECs are *costimulatory* molecules, representing the second signal transduction pathway in Ag presentation (also known as accessory pathway), which is certainly essential for T-cell activation. Several studies described the expression of costimulatory molecules on IECs: it was observed that CD58, but not CD80 (a ligands for CD28 on T cells) or CD86 (a ligand for CTLA4) is constitutively expressed on both native IECs and in the IEC lines T84 and HT29. The surface expression of CD58 (LFA-3) was highly polarized and restricted to the basolateral surface of IECs (101). As mentioned above, the conventional costimulatory molecules CD80 (B7.1) and CD86 (B7.2) are not constitutively expressed on IECs, except in inflammatory circumstances. The correspondent receptor on T cells for CD80 and CD86 molecules is CD28. CD80 and CD86 messenger RNA was detected in isolated colonic epithelial cells from normal and inflamed mucosa of patients with UC, while the immunohistochemistry and flow cytometric analysis of colonic epithelial cells from inflamed colonic mucosa confirmed the expression only for CD86. In the same time, cell surface expression of CD86 protein was increased after IFN-γ stimulation of the colonic epithelial cell line HT29 (102).

From the novel B.7 costimulatory family molecules, B7h- and B7-H1-messenger RNA was also detected both in IEC lines and IECs from healthy controls and patients with IBD. IECs from patients with IBD, but not healthy controls expressed B7h and B7-H1 protein on their surface. Proliferation of IEC-stimulated T cells (in co-cultures) was inhibited only by B7h immunoglobulin treatment, suggesting that only the B7h-ICOS costimulatory pathway may be important in IEC-T cell interactions (103).

Beside CD58, the expression of intercellular adhesion molecule-1 (ICAM-1 or CD54, the receptor for LFA-1 in IECs was also described (104).

Last, but not least, the hallmark of costimulatory molecules in the APC-T cell interaction is the CD40-CD40L system, that amplifies the immune response and can promote inflammation (79). Bataglia and colleagues have brought the evidence for local accumulation of $CD40L^+$ together with $CD40^+$ cells within intestinal lesions of CD, suggests the involvement of this costimulatory pathway. Basically, in the absence of secondary signals, resting T cells develop unresponsiveness (anergy) or tolerance to antigenic stimulation (4).

Additional immune competence of IECs was the identification of IECs' ability to produce potent immunoactive components, like cytokines and chemokines, both

constitutively and after invasion with bacterial pathogens, being important for the initiation and amplification of an acute mucosal inflammatory response. IECs from established epithelial cell lines like T84, Caco-2, SW620, and HT29 constitutively secrete interleukine 8 (IL-8, a potent chemoatractant for neutrophils), transforming growth factor beta 1 (TGF-ß1), the monocyte chemotactic protein-1 (MCP-1), tumor necrosis factor alpha (TNFα) and granulocyte-macrophage colony stimulating factor (GM-CSF); IL-1α, IL-1β, IL-10 was partially also detected. None of the cell lines expressed mRNA for IL-2, IL-4, IL-5, IL-6, or INF-γ (8, 105). Beside the endogenous cytokine production, IEC lines can also respond to a broad array of cytokines e.g., TNFα, IL-I, IL-4, IL-6, IFN-γ (8, 105-107). These cytokines of either autocrine or paracrine origin may form a cascade or cytokine network, regulating the APC function of IECs, as well as other cells in the intestine. Furthermore, IECs express complement proteins (C3, C4, Factor B) and leukotrienes, suggested to be involved in the initiation and regulation of mucosal inflammation (108, 109).

As conclusive idea of this chapter, it is clear that IECs participate in inflammatory processes, but directed functional *in vivo* studies will be required to address their true role in the gut immunoregulation.

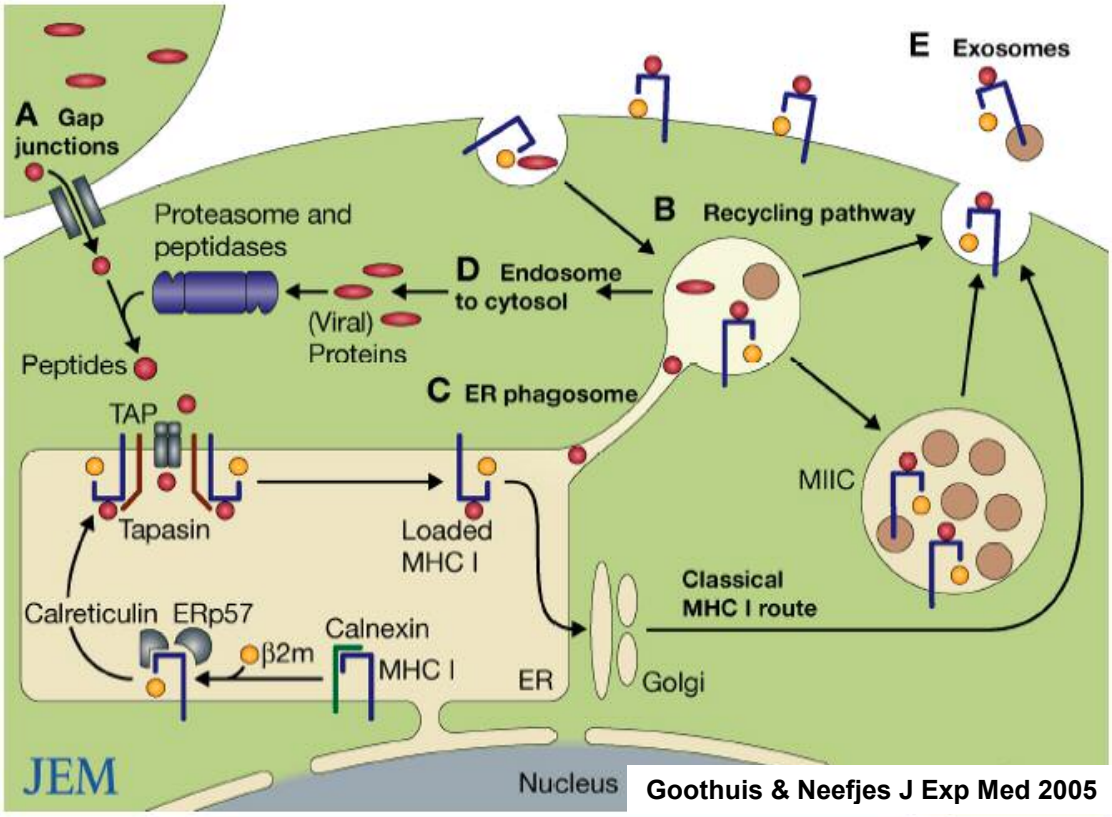
Cross-presentation

Beside the classical dihotomic Ag presentation of exogenous Ags via MHC II to $CD4^+$ T cells and endogenous Ags via MHC I to $CD8^+$ T cells, there is an additional Ag presentation process with great importance in tolerance and immunity, the so-called “cross-presentation”. As a simplified definition, cross-presentation implies presentation of exogenous Ags via MHC I towards $CD8^+$ T cells and endogenous Ags via MHC II to $CD4^+$ T cells. MHC class I and class II molecules sample antigenic information from different sources, both intracellular and extracellular. A major exception occurs during cross-presentation: it can involve either class I- or class II-restricted Ags. This is an important aspect, since initial cross-presentation experiments involved class-I restricted cytotoxic T cells priming to cell-associated Ags. T cell activation resulting from cross-presentation leads to T cell priming (cross priming) or T cell tolerance (cross-tolerance) (110-112).

DCs and macrophages are the principal cells that cross-present exogenous Ags *in vitro* and *in vivo* (38, 110, 113-116). Still, the exact mechanisms of cross-presentation are not yet completely understood and defined. *In vivo*, DCs, the major cell type

responsible for cross-priming, acquire endogenous Ags for instance from infected cells in the periphery. Then they migrate to the lymph nodes where the antigenic peptides are displayed in association with MHC class I molecules. MHC class I–peptide complexes are recognized by Ag-specific cytotoxic T lymphocytes, which become activated and expand in response to Ag recognition (117).

Several experiments have shown that extracellular proteins can be transferred from endosomes into the cytosol of DCs (118), although how this occurs is still unclear. It might involve dissolution of the endocytic membrane or specific protein transporters that pump the Ag out of endosomes and/or Lys. This implies a solubilization of an Ag-containing endocytic structure, which would liberate endosomal proteases and likely result in the autolysis of the cross-presenting cell (112).



Scheme 3. ***Different models for cross-presentation.*** The classical MHC class I Ag–presenting pathway implies that intracellular Ags are degraded by the proteasome and peptidases. A fragment of the resulting peptides binds to transport associated peptides (TAP) in the endoplasmic reticulum (ER) membrane where newly synthesized MHC class I molecules are arrested until complexed with peptides. MHC class I–peptide complexes then leave the ER and are translocated to the plasma membrane. There are different suggested pathways for Ag entering. (A) Gap junctions permit direct transfer of peptides from infected cells into the cytosol of DCs. (B) MHC class I molecules can enter the recycling pathway and exchange peptides. (C) ER components become part of the

phagosomal pathway. The ER-associated degradation system pathway then exports exogenous Ag from the phagosome into the cytosol and phagosomal TAP allows revers-transport of peptides back into the phagosome. (D) Exogenous Ags can be transported over the endosomal membrane. (E) Exosomes secreted by APCs can bind to DCs for cross-presentation (from (112).

Another possibility could be that MHC class I molecules can be recycled from the cell surface along the endocytic class II pathway and exchange endogenous for exogenous peptides on the way (63).
Recent studies revealed a role for a supposed endocytosis signal in MHC class I (119) and for endosomal proteases (111) in cross-presentation, which supports a role for the recycling pathway. In this model, Ags would be degraded by endocytic proteases, rather than the proteasome. Thus, some Ags that would normally be presented in the classical pathway might not survive to be cross-presented.
Exosomes are Ag presenting vesicles derived from the interior of endocytic structures. They are released by many cell types and can also induce $CD8^+$ T cells responses by cross-presentation (120). How these small vesicles, which contain MHC class I–peptide complexes, trigger $CD8^+$ T cells activation is unclear. In addition, various experiments have shown that extracellular proteins can be transferred from endosomes into the cytosol of DCs (118), although how this occurs is not clearly described yet.

Oral tolerance

The oral tolerance is well characterized as being a form of immunological tolerance, in which the feeding of a “non self” Ag leads to a specific and active immunological unresponsivenes, like in the case of food Ags. There are three major mechanisms in oral tolerance: clonal anergy, deletion of effector T cells and induction of suppressor T cells. *Clonal deletion* is explained by a deletion of Ag-specific T cells by the induction of apoptosis (a form of cell death that occurs by the activation of a internal cellular death program). This phenomenon has been observed in experimental animal models as an immune response to a verry high (nonphysiological) quantity of oral Ag (2). Deletion is rarely found in peripheral tolerance to nominal Ags, and has not been described yet in tolerance induction in normal animals (121). Studies in T cell receptor-transgenic mice have proved that high dosis of Ags (like OVA) can

induce Ag-specific deletion of T cells in the spleen and GALT of Ag-fed mice (122, 123).

High doses of Ag fed to normal mice are believed to induce clonal *anergy* (123, 124). A circumstantial evidence for clonal anergy is the failure to transfer tolerance and the ability to rescue the function of tolerized lymphocytes by administration of IL-2 (124, 125). The third tolerogenic response is the development of regulatory T cells, which are able to suppress actively an Ag-specific response.

Further studies indicated that $CD4^+$ T cells, rather then $CD8^+$ T cells, are required for oral tolerance induction (126-128). Active suppressor mechanism can induce the "*bystander effect*", in which tolerized T cells secrete a suppressive cytokine, like TGF-β (121). Induction of bystander effect and more generally oral tolerance, has a fundamental therapeutic importance. Oral tolerance could be eventually used in treating autoimmune diseases via the mucosal route, by administering the specific Ag, if this one is identified (124).

There are several factors that enhance the induction of oral tolerance, like IL-4, IL-10, anti-IL-12, TGF-β, cholera toxin B subunit and anti-CD40 ligand. Oral (and nasal) Ag administration suppresses autoimmune diseases in animal models of autoimmune encephalitis, uveitis, thyroiditis, myasthenia, arthritis, and diabetes (in the non-obese diabetic mouse), as well as non-autoimmune diseases, such as asthma, atherosclerosis, graft rejection, allergy, colitis, stroke, and experimental Alzheimer's disease). Oral tolerance has been tested also in human diseases including multiple sclerosis, arthritis, uveitis, and diabetes, contact sensitivity to dinitrochlorobenzene and nickel allergy. Although positive results have been observed in phase II trials, no effect was observed in phase III trials of type II for collagen in rheumatoid arthritis, or oral myelin and glatiramer acetate in multiple sclerosis (129).

A potential defect in gut immunoregulation has also been suggested to contribute to the pathogenesis of IBD, as a consequence of the loss or break down of tolerance towards the gut flora and/or food Ags (130). Whether patients with IBD have an underlying defect in oral tolerance has not been clearly defined yet, as functional (prospective) studies to investigate these defects are difficult to perform in humans. However, a non-prospective study on regulatory responses was carried out in CD patients, in which T cells were extracted and incubated *in vitro* with the own tolerized gut microflora. In this study, the authors proved that T cells from CD patients proliferate and produce cytokines when co-incubated *in vitro* with extracts of own

microflora, while cells from controls do not (130). This important experiment indicated that a loss of tolerance to Ags in the mucosal microflora occurs in IBD, and in the same time an excessive response to own Ags.

Mucosal tolerance, however, remains an attractive approach for treatment of autoimmune and inflammatory diseases because of complete absence of toxicity, easy administration over time, and Ag-specific mechanisms of action. The successful treatment of human diseases by oral tolerance will eventually depend on developing immune markers to assess immune effects, dose and the chosen way of administring (nasal versus oral) (129).

What is inflammatory bowel disease?

CD and ulcerative colitis (UC), together referred to as IBD, are complex chronic intestinal disorders of idiopatic-multifactorial origin, related to a not yet entirely understood interplay of genetic, immunologic, infectious, allergenic and psychogenic factors leading to a longterm, relapsing and sometimes irreversible damage of the gastrointestinal structures and function (131). Despite incomplete understanding of IBD, it remains a fascinating group of diseases, a continuous challenge for the gastroenterological and immunological researcher community.

CD was first described 1932 by Dr. Burill Crohn and colleagues. At that time, it was believed to be an infectious disease, comparable to mycobacterial infections of the gut (132). This concept was, however, outdated in the 1970s, when the knowledge about cytokines, lymphocytes and monoclonal antibodies revolutionized the perception on the role of mucosal immunology in IBD etiopathogenicity.

Recent works in animal models, human genetics, basic science and clinical trials have provided new insights into the pathogenesis of these enigmatic diseases. The current, most widely spread hypothesis on the IBD etiopathogenesis is that in a genetically predisposed host there is an acquired aberrant and in the same time aggressive T cell immune response to a subset of the commensal bacterial flora and that environmental factors trigger the onset or reactivation of the disease.

The two main forms of IBD, CD and UC, are chronic immune mediated diseases, having similarities but also several distinguishable clinical and pathological features.

In a minority of cases, the two entities are indistinguishable and termed “indeterminate colitis”.

General features of Crohn's disease

The prevalence of CD ranges between 10-200 cases/100.000 individuals in North America and Europe. The highest disease incidence is in the urbanized and developed countries. IBD is becoming more common also in the rest of the world, as more countries are developing a western lifestyle (133). The disease can affect any age group, but the onset or diagnosis occurs mostly in the second and third decade (teenagers and young adults). CD can affect any part of the gastrointestinal tract, most commonly the terminal ileum, cecum, the perianal region and colon, in so-called "skip lesions" (131).

The clinical features of CD include diarrhea, pain, bowel strictures, obstructions, abscesses and various fistulization. IBD has an increased risk of carcinogenesis, in CD being less frequent compared to longstanding UC. Extra-intestinal manifestations include affection of skin, joints, eye and liver. From histological point of view, CD affects all layers of the intestinal wall and is characterized by lymphocytic and macrophages infiltration, presence of granuloma (approx. 60%), fissuring ulcerations and submucosal fibrosis (134).

The current treatment of CD includes: corticosteroids, azathioprine, 5-aminosalicylic acid, methotrexate, as well as modern cytokine based therapies (like antibodies against TNFα: Infliximab, Adalimumab) and bowel-sparing surgical resection of obstructed enteric segments (131, 134, 135).

Aim of the study

The hypothesis of the present study was that IECs represent a key player in CD pathogenesis, being involved in the inflammatory cascade through a pathological stimulation of T cells. Thus, the modulation of intracellular Ag trafficking mechanisms and consecutively Ag processing within IECs could lead to the upregulation of costimulatory molecules and consecutive to an aberrant Ag presentation by IECs.
Since MHC I and MHC II molecules are the major regulators of Ag presentation, the aims of this study were:
1) to describe the extracellular and intracellular MHC class I and class II expression, *in vivo*, in ileal epithelial cells and to identify possible differences between healthy and CD patients, both qualitatively and quantitatively.
2) to identify the subcellular compartments of IECs involved in MHC I- and MHC II-related processing and presentation of exogenous Ags.
3) to characterize the precise trafficking pathways of exogenous Ags with special regard to the transport timing and the possible inflammation-related kinetic differences.
4) to identify, *in vivo*, IECs` derived exosome-like vesicles within the intercellular spaces, since exosome-like vesicles were described only in mice and human IEC lines so far.
To achieve these goals, I have used a prototypical soluble protein, OVA by the fashion of an already established technique **(Scheme 4)** (100) and studied the epithelial trafficking of this exogenous Ag. Ag (OVA) exposure proceeded on healthy and inflamed mucosa, during ileoscopies. Regarding the issue of differential capacity of IECs to function as APCs, which might rely on differences in intracellular Ag sorting and MHC molecule expression, I've focused on the small bowel of CD patients and healthy controls.
These results yield first *in vivo* insight into the transport of luminal Ags in human ileal IECs and its linkage with MHC I and MHC II pathways. It was of high relevance to investigate the endocytic system of IECs, especially to explore in detail the site of encounter of endocytosed materials with MHC molecules, describing also human IECs'-derived (potential) Ag-presenting vesicles, known as exosomes.

Materials and Methods

Principles of immunofluorescence and confocal microscopy (136-138)

Fluorescence microscopy is a very popular scientific and diagnostic tool with large application in the biomedical field, both in reseach and diagnostics. Like the majority of immunological tools, immunofluorescence (IF) is based on the Ag-antibody reaction, meaning that antibodies bind specifically to their corresponding Ag, giving the possibility to identify and locate a particular molecule in tissue sections or cells. In IF, certain antibody molecules can be used to target the corresponding Ag in a specific section, by using a fluorescent dye. Before applying the dye (labeling), the native structures (receptors) intented to be detected within the tissue sample need to be preserved by using frozen tissue sections that are fixed only after the antibody reaction. Antibody stably binds to its Ag, allowing unbound antibody to be removed by successive washing steps. In the *indirect IF*, the bound (primary) antibody is detected by a fluorescent anti-immunoglobulin (secondary antibody), while in the *direct IF* the fluorescent dye is coupled directly on the primary antibody.

Basically, the dyes chosen for IF are excited by light of one wavelength, usually blue or green, and emit light of a different wavelength in the visible spectrum. All parts of a specimen throughout the optical path of the microscope will be excited by the light source and the emited fluorescence will be detected by a photodetector, so that the investigator can visualize it on the conventional ocular device of the IF microscope, and/or alternatively on a IF camera connected to a PC/monitor.

Confocal microscopy is an optical imaging technique that uses the same specimens as conventional fluorescence. Compared to the conventional IF microscope, it has major advances since it allows one to visualize not only deeper into cells and tissues structures, but also to create images in 3D. The principle of confocal microscopy uses an increased micrograph contrast and reconstructs three-dimensional images. In contrast to conventional fluorescence, a confocal microscope is a more versatile instrument using point illumination from a laser light source and a pinhole in an optically conjugated plane (in front of the detector) to eliminate out of focus information. By removing the “out of focus” light, only the light within the focal plane is detected, resulting in a image of exceptional resolution and clarity, of a higher quality than conventional fluorescence. 2D or 3D imaging reconstruction requires scanning over a regular raster in the specimen, involving changing the level or plane at which

the sample is observed. A computer is used to store the intensity value of each point from the detector, and presents these in the correct order on a high resolution video monitor to display the image. In conclusion, there are at least 3 major advantages of confocal upon conventional IF microscopy:

- it produces images of improved resolution, up to 1.4 times higher than standard microscopy, by eliminating out of focus light.
- it has a higher level of sensitivity compared to conventional microscopes, because of highly sensitive light detectors.
- it has the ability to produce 3D reconstructions of specimens.

Principles of immunoelectron microscopy (2, 136, 139)

Conventional electron microscopy is an important tool for nano-technologies and biomedical research and development. It uses electrons (subatomic particle with negative electric charge) to create an image from a specimen. It has higher magnification (up to about 2 million times) than the classical light microscope (max. 2.000), allowing to detect smaller objects and greater details in the tissue samples. Instead of glass lenses to focus light (as the light microscope), the electron microscope uses electrostatic and electromagnetic lenses to focus the image. The principle of transmission electron microscopy (TEM) bases on a high voltage electron beam emitted by a cathode and focused by electrostatic and electromagnetic lenses. The electron beam transmitted through the ultrathin specimen or section “takes” the information of the specimen or structure in the electron beam, reaching the imaging system of the microscope. The spatial variation of the transpassed information is magnified by a series of electromagnetic lenses and projected on a fluorescent screen and/or photographic plate or camera. Among the several subtypes of electron microscopy, the immunoelectron microscopy (IEM) has the largest application in clinical medicine (e.g. in diagnostic histopathology) and research. Actually, it is an indispensable technique to elucidate at high resolution the intracellular localization of manifold biologically active substances or particular proteins. Based on the principle of the conventional electrone microscopy, this technique is basically the same as that of light microscopic immunohistochemistry. One of the prerequisites is that the markers used in the immunostaining have to be electron-dense, in order to be visualized. Antibodies against the required Ag are labeled with colloidal gold particles of different sizes (5, 10, 15, or 20 nm) giving the possibility to study two ore more

proteins simultaneously. The immunogold stainig is done on ultrathin sections, in which the interior of the cut tissue or cells (with the organells) is exposed on the cut surface. The section surface enables labeled antibodies to easily reach the suitable Agic sites. Immunostained ultrathin section can be examined at the transmission electron microscope.

Patients

The present study included 47 subjects in a total of 24 CD patients (12 male, 12 females), and 13 controls (6 male, 7 females). All 47 underwent ileocolonoscopy. The median age of CD patients was 39 (range 25-58) and 59 for controls (range 24-85). CD patients were recruited on the basis of ileal disease, either active ileitis (n=13) or ileitis in remission (n=11). Patients undergoing ileocolonoscopy for carcinoma screening, gastrointestinal bleeding, constipation or abdominal pain served as controls. The severity of IBD was assessed by clinical, endoscopical, histopathological and serological criteria. CD patients in remission and controls had no signs of any bowel or systemic inflammation. Clinical history did not indicate any food intolerance or allergy. Infectious ileitis was excluded by stool culture and serology. CD patients' specific medication consisted of corticosteroids (n=8), azathioprine (n=10), mesalazine (n=10), sulfasalazine (n=2), cyclophosphamide (n=1) and methotrexate (n=1). None of the control patients received immunosuppressive medication.

Ethics

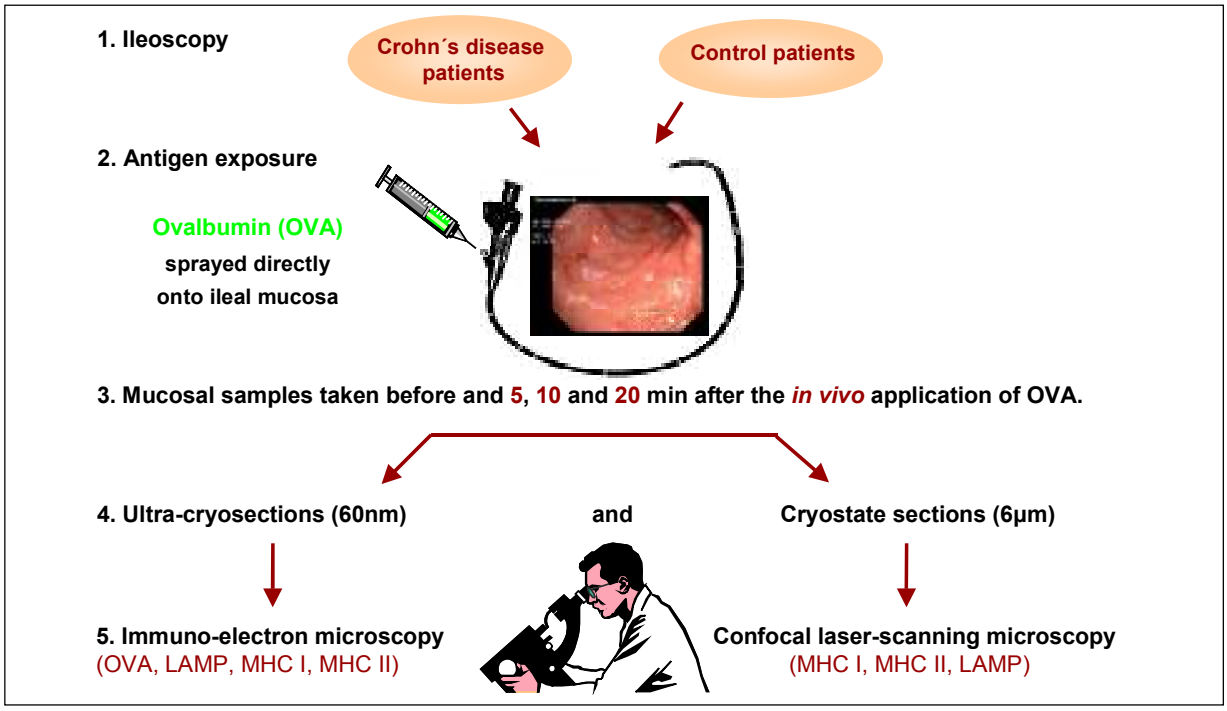
All subjects gave their written informed consent regarding the participation at the study. In addition, the study was approved by the Ethics Committee of the Medical Faculty, University of Lübeck (No. 02-073 and 03-043) and the German National Drug Administration (BfArM: 4021154) and was conducted according to the Declaration of Helsinki.

Tissue sampling and antigen exposure to ileal mucosa

For IF investigation and resin-embedding, specimens of ileal mucosa (size 5 mm) were obtained endoscopically. Biopsies were taken within the terminal ileum from macroscopic inflamed mucosa in CD ileitis and normal mucosa in CD patients in

remission and controls. Histopathological examination confirmed granulomatous CD-specific inflammation and healed or healthy mucosa, respectively.

For immunoelectron microsopy, *in vivo* exposure of OVA to the ileal mucosa was performed in patients, which consented to this supplemental procedure (CD ileitis n=5, CD ileitis in remission n=3, controls n=4). During endoscopy, 10 mL of OVA solution (100 mg/mL in saline; fraction V, Sigma, Taufkirchen, Germany) was sprayed directly onto saline cleaned inflamed or healthy ileal mucosa. Biopsies were obtained before and after 5, 10 and 20 minutes of OVA incubation. For ethical reason, mucosal OVA incubation for 60 minutes was in part carried out in biopsies *ex vivo*. Biopsies taken 20 minutes after *in vivo* exposure, were immediately transferred into cell culture medium (DMEM containing 10 % foetal calf serum, PAA, Pasching, Austria) and kept there for another 40 minutes at 37°C and 5 % CO_2.



Scheme 4. ***Comprehensive scheme of the methods used in this study.***

During ileoscopy, ovalbumin (OVA) was sprayed directly onto ileal mucosa of CD patients (ileitis and remission) and controls. Tissue samples were extracted and prepared for fluorescence light and electron microscopy.

Antibodies

The following primary antibodies were used: affinity-purified polyclonal rabbit antibodies against OVA (98, 100) and against MHC I and MHC II (both a gift from J. J. Neefjes, National Cancer Institute, Amsterdam, The Netherlands) (39); monoclonal

mouse antibodies against MHC I (clone HC10, gift from J. J. Neefjes) (140), against MHC II (clone CR3/43, gift from J. Cordell, John Radcliffe Hospital Headington, Oxford, UK) (141), and lysosome-associated membrane protein-2 (LAMP-2, clone H4B4, BD Bioscience PharMingen, Hamburg, Germany).

Binding sites of primary antibodies were visualized by electron microscopy using gold-conjugated goat antisera against rabbit and mouse IgG (6 or 12 nm in diameter; Dianova, Hamburg, Germany). For IF, goat antisera against rabbit and mouse IgG conjugated to Alexa-Fluor 488 and 555 (Molecular Probes, Eugene, OR) were used. OVA antibodies did not cause non-specific labeling on sections from ileal mucosa solely exposed to saline. Our group has previously demonstrated the specificity of the polyclonal OVA antibodies by preincubation of OVA antibodies with OVA (142).

Tissue preparation and labeling for immunofluorescence and confocal microscopical investigation

Ileal specimens were frozen in liquid nitrogen. Cryostate sections (approx. 6 µm in thickness) were cut at –20°C, mounted on glass slides and air-dried over night. After fixation in acetone/methanol (1:1), the cryosections were successively incubated with primary antibodies, appropriate Alexa Fluor-conjugates (Molecular Probes) and bis-benzimide (Sigma), for 60 minutes each. Double-labeling was performed simultaneously, using primary antibodies derived from different species and appropriate fluorochrome-conjugates. Cross-reactivity of antibodies applied in double-labeling experiments was excluded. IF analysis was performed using a Zeiss LSM 510 Meta confocal microscope (Jena, Germany).

Tissue preparation and labeling for immunoelectron microscopical investigation

In ordet to be labeled and analyzed by electron microsocopy, the biological material has to be sliced into ultrathin sections, by the method of cryosectioning (sectioning at very low, cryogenic temperatures, maintained by liquide nitrogen).

The cryo sectioning and labeling experiments of ultrathin sections for immunoelectron microspopical study, were carried out according to published protocols (post-embedding technique of Tokuyasu and Griffiths (143). Briefly, biopsies of ileal mucosa were fixed in 5% formaldehyde, followed by cryo protection in 0.03 M polyvinylpyrrolidone/1.6 M sucrose, to prevent ice crystal damage to the biological

material. These steps were followed by ultra rapid refrigeration in liquid nitrogen at –196°C, by the *snap-freezing method* (plunging a specimen directly into a beaker of liquid nitrogen). Long-term tissue storage and preservation was done in liquid nitrogen storage vessels. Cryogenic liquid nitrogen storage (cryoconservation), is regarded as the most reliable method to preserve cellular and ultrastructural integrity (including the antigenic/proteic components) for electron microscopic analysis (144). Ultrathin cryosections (approx. 60 nm in thickness) were cut using a Leica ultracryotome at – 110°C, mounted on formvar coated copper grids and consecutively incubated with primary and species-specific gold-conjugated secondary antibodies for 45 minutes each. Labeled grids were contrasted with uranyl acetate, embedded in 2 % methylcellulose and analyzed using a Philips EM 400 T transmission electron microscope (Kassel, Germany).

Double-labeling experiments were performed applying primary antibodies from different species and corresponding 6 and 12 nm immunogold-conjugates, respectively. Double-labeling was carried out for MHC I / LAMP-2, MHC II / LAMP-2, MHC I / MHC II, OVA / LAMP-2 and OVA / MHC I. Possible cross-reactivity of antibodies was excluded for each double-labeling step. Double-labeling for OVA / MHC II could not be performed due to inefficacy of the monoclonal anti-MHC II antibodies available in immuno-labeling of ultrathin cryosections.

Electron microscopy of resin-embedded sections

Specimens of ileal mucosa were fixed in 2 % glutaraldehyd / 0.6 % formaldehyde in 0.06 M sodium cacodylate / HCl buffer (pH 7.3). After post-fixation in OsO_4, tissues were dehydrated in graded ethanol solutions and embedded in Araldite M (Fluka, Neu-Ulm, Germany). Ultrathin sections (60 nm in thickness) were prepared, stained with uranyl acetate and lead citrate, and examined using a Philips EM 400 T transmission electron microscope.

Quantitation of MHC I and MHC II labeling in IECs

The relative distribution of the subcellular labeling for MHC I and MHC II in IECs was assessed separately in 9 randomly chosen patients (3 patients for each group: CD ileitis, CD ileitis in remission and controls). Quantitation was done by an observer unaware of any clinical information and carried out according to the method introduced by Lucocq et al. (145). Ultrathin sections were double-labeled for MHC

I/LAMP-2 and MHC II/LAMP-2, respectively. On one randomly taken grid per patient, 400 gold particles representing binding sites for MHC I or MHC II were counted and assigned to the different subcellular compartments within IECs. According to previous work on professional APCs, compartments were identified due to their ultrastructural morphology and specific marker proteins (e.g. LAMP-2) (43, 146). In line with Lucocq et al. (145), the quantitation of 400 gold particles per Ag on one grid is evaluated with an error coefficient of up to 5% and considered sufficient for statistical validation. The percentage of gold counts for each compartment was established per Ag and patient. An arithmetic mean of the percentage was calculated for each patient group and Ag used, in order to elaborate a ranking of the labeling distribution for MHC I and MHC II **(Fig. 4 and 5)**.

Individual research results

MHC I and MHC II expression in ileal epithelial cells of Crohn's disease patients and healthy controls

Since MHC molecules are the major Ag processing and presenting regulators, one of the main goals was to explore and describe the expression of MHC I and MHC II molecules within IECs. The IF experiments using monoclonal antibodies against MHC I and MHC II revealed regional differences in the epithelial expression, depending on the degree of mucosal inflammation **(Fig. 1)**. Staining for MHC I and MHC II was observed in the entire ileal epithelium, both in IECs and cells of the LP. In IECs, both MHC I and MHC II were predominantly detected on the basolateral membrane and within cytoplasmic granules. Apical membranes of IECs revealed faint or no staining for MHC I and MHC II. MHC I was found in crypt and villus of IECs **(Fig. 1 A, B, and C)**, regardless of mucosal inflammation status. In contrast to MHC I molecules, inflammation influenced the staining pattern for MHC II along the crypt-villus axis. A strong expression was constantly observed in crypts of inflamed mucosa **(Fig. 1 F)**, while staining in crypt IECs was absent or faint in healthy mucosa of controls and CD ileitis in remission **(Fig. 1 D, E)**. Villus IECs showed consistent MHC II expression throughout all patient groups.

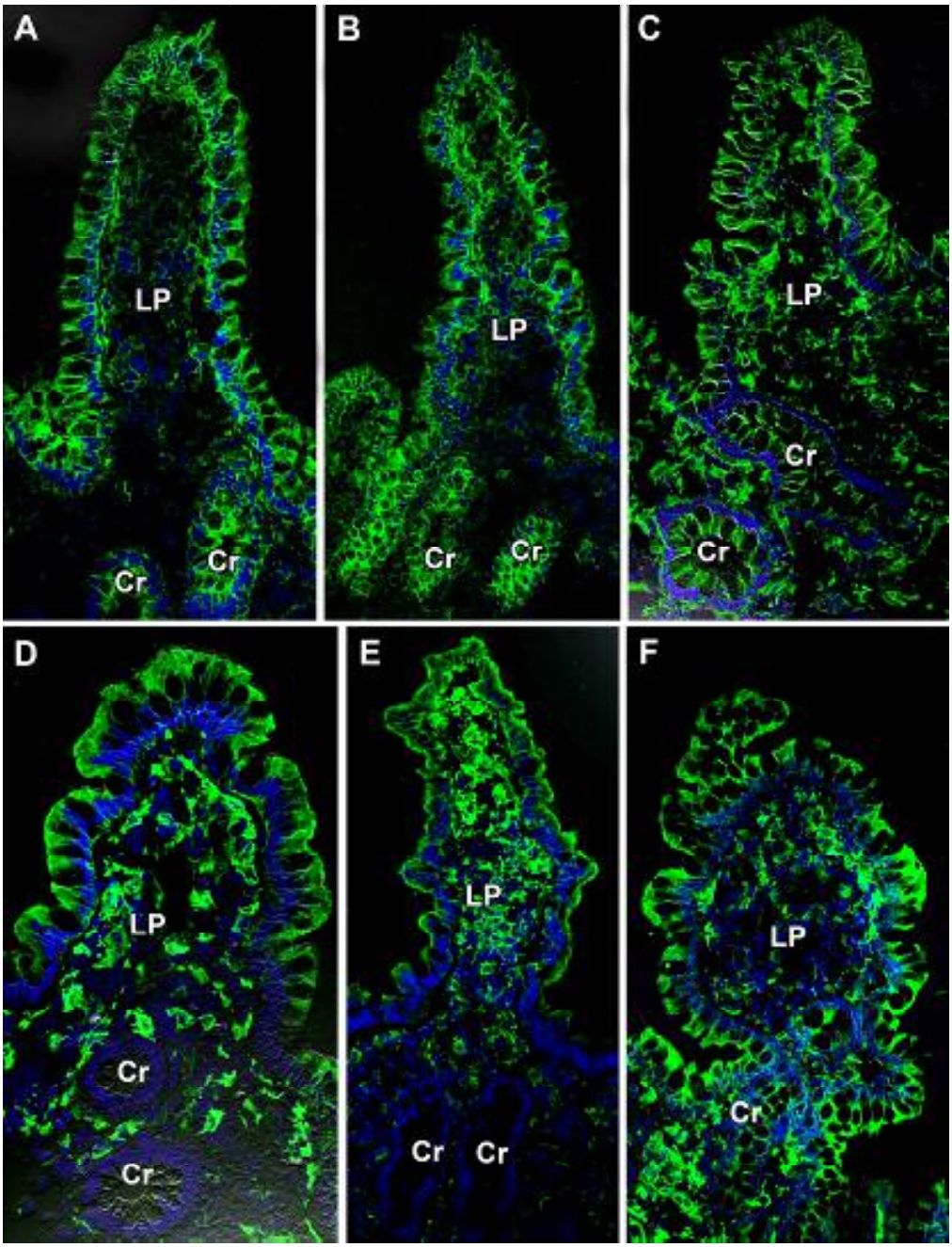


Fig. 1. ***Expression of MHC I and MHC II in the ileal mucosa of CD patients and healthy controls.*** MHC I **(A, B,** and **C)** and MHC II Ags **(D, E,** and **F)** are visualized by green fluorescence on cryosections. The blue fluorescence stained nuclei. Ileal mucosa is taken from a control **(A, D)**, a CD patient in remission **(B, E)** and a CD ileitis patient **(C, F)**. MHC I is found in villus and crypt (Cr) IECs, independently of the inflammatory state of the mucosa. In contrast, epithelial staining for MHC II in the non-inflamed mucosa of healthy controls **(D)** and CD patients in remission **(E)** is restricted to villi. In CD ileitis **(F)**, both villus and crypt IECs show staining for MHC II. Cells of the lamina propria (LP) reveal MHC I and MHC II in all patient groups.

After the detailed visualization of MHC molecules distribution by simple IF stainig, double-labelings were performed to further dissect the precise subcellular nature of

the granular staining for MHC I and MHC II in IECs, and to confirm the assumed late endocytic origin. In these double-labeling experiments, LAMP-2 and either MHC I or MHC II were simultaneously visualized. Merged confocal images of double-labeled sections consistently revealed co-localization for LAMP-2 / MHC I **(Fig. 2)** and LAMP-2 / MHC II **(Fig. 3)** in IECs. Co-localization was predominantly found in the supranuclear part of IECs and indicated the presence of MHC I and MHC II molecules within late endocytic structures of IECs. Beyond this, some intracellular staining in particular for MHC I and to a lesser extent for MHC II was seen in LAMP-2 negative regions. MHC I and LAMP-2 co-localized within crypt and villus IECs regardless of the degree of mucosal inflammation **(Fig. 2)**. Our results obtained with the monoclonal antibodies against MHC II, co-localization for MHC II and LAMP-2 in crypt IECs was restricted to inflamed mucosa, while in villus IECs it was present in all subjects **(Fig. 3)**.

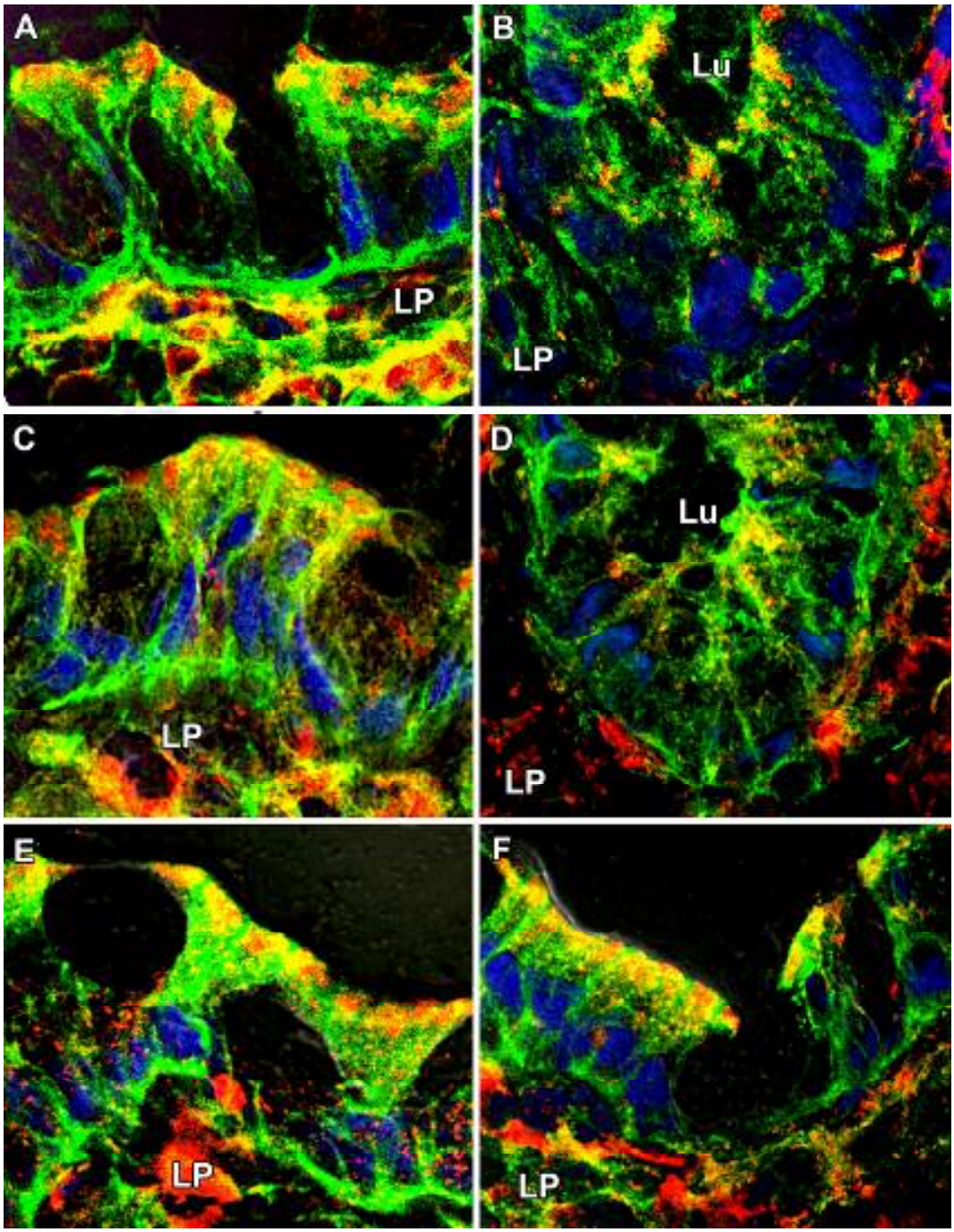


Fig. 2. ***MHC I resides in late endocytic compartments of IECs.*** MHC I (green fluorescence) and LAMP-2 (red fluorescence) are double-labeled on cryosections. Sections are made from ileal mucosa obtained from a control **(A, B)**, a CD in remission **(C, D)** and a CD ileitis patient **(E, F)**. The merged images show co-localization (yellow fluorescence) for MHC I and LAMP-2 in the supranuclear part of IECs. Cell surface staining for MHC I in IECs is predominantly seen at the basolateral membranes. No differences are found regarding villus **(A, C, E)** and crypt **(B, D, F)** IECs and this is not dependent on mucosal inflammation. Co-localization of MHC I and LAMP-2 is additionally detected in cells of the lamina propria (LP). Lu, lumen.

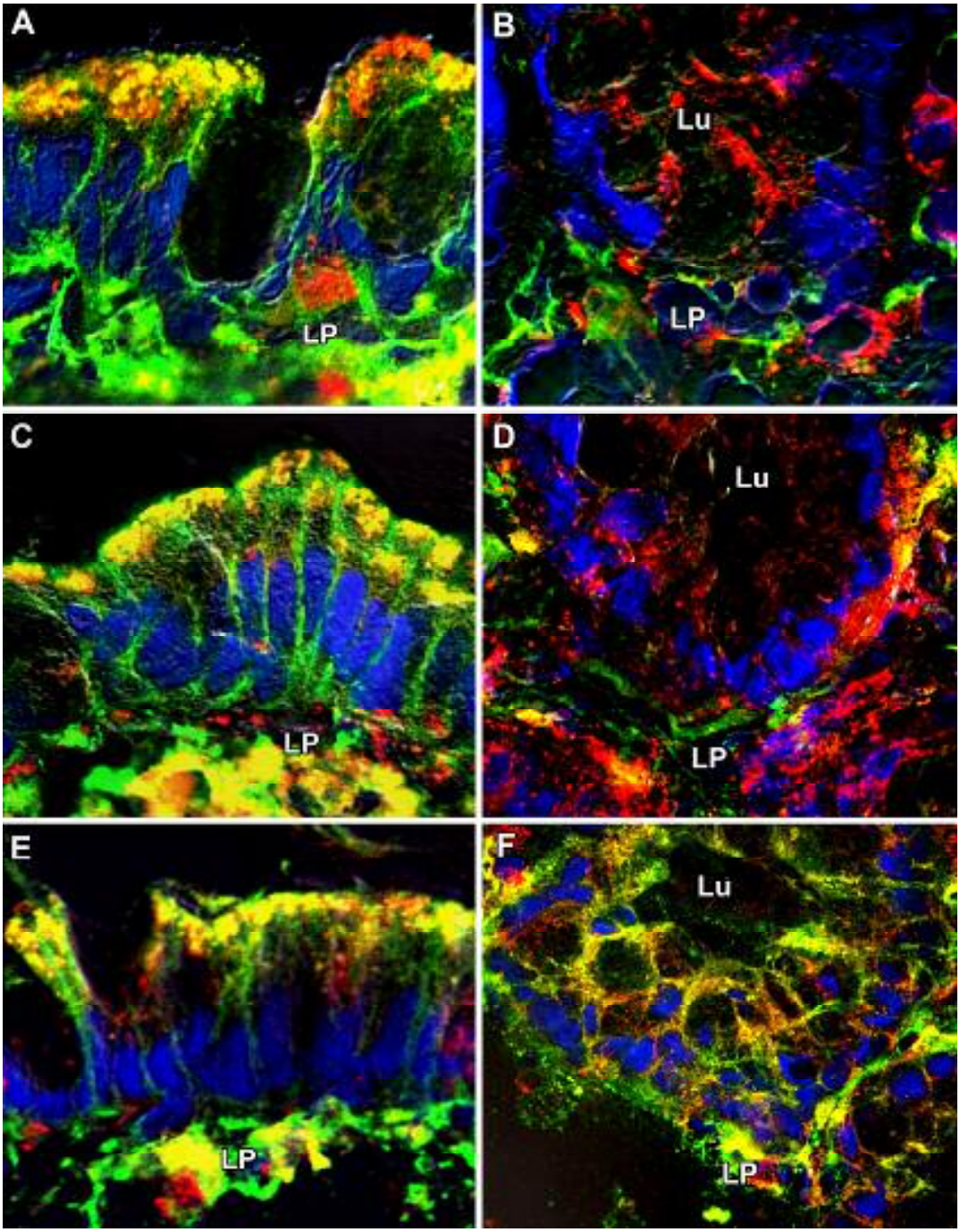


Fig. 3. ***Intracellular MHC II accumulates in late endocytic compartments of IECs.*** The merged images show a double-staining for MHC II (green fluorescence) and LAMP-2 (red fluorescence) on cryosections. Biopsies from ileal mucosa are taken from a control **(A, B)**, a CD in remission **(C, D)** and a CD ileitis patient **(E, F)**. Co-localization of MHC II and LAMP-2 (yellow fluorescence) is observed in the supranuclear part of villus IECs **(A, C, E)** constantly. While crypt IECs from controls **(B)** and CD patients in remission **(D)** lack staining for MHC II, crypt IECs affected by CD ileitis show co-localization for MHC II and LAMP-2. Cell surface staining of MHC II in IECs is mainly observed on basolateral membranes. Cells of the lamina propria (LP) revealed MHC II / LAMP-2 co-localization in all patients. Lu, lumen.

The majority of MHC I and MHC II molcules accumulates within late endosomes of the endocytic system of intestinal epithelial cells

After decribing the epithelial MHC I and MHC II expression within IECs, both by simple and double-labeling IF techniques, in order to identify and characterize the morphology of the cellular compartments involved in co-localization of MHC I/II and LAMP-2, I performed double-labeling cryo-immunoelectron experiments on ultrathin frozen sections. The subcellular distribution of immunogold-labeling for MHC I **(Fig. 4)** and MHC II **(Fig. 5)** was quantitatively assessed to characterize the pathways of these molecules in IECs.

Cell surface labeling of MHC I and MHC II in IECs was predominatly seen at basolateral membranes (~41% and ~33% respectively). Weak labeling for both MHC molecules was detected on the apical membrane (including microvilli) (~4%for MHC I and ~8% for MHC II). All components of the endocytic tract revealed labeling for MHC I and MHC II, as distributively depicted below.

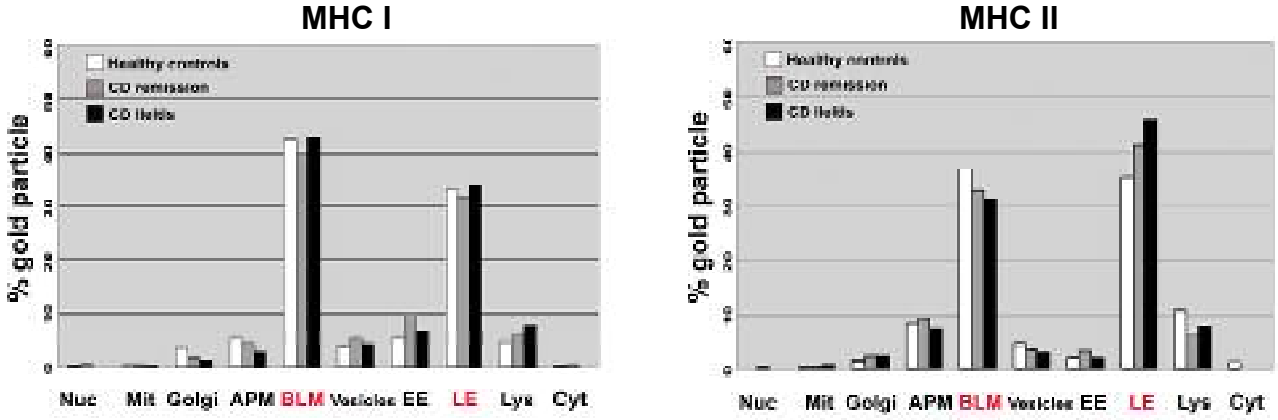


Fig. 4 and 5. ***Quantitative analysis of immunogold labelings on ultrathin cryosections.*** Intracellular localization of MHC I and MHC II predominate in late endosomes (LE~32% and ~40%). Cell surface labeling is mainly found at the basolateral membranes (BLM~41% and ~33%). Nuc, nucleus; Mit, mitochondria; APM, apical membrane; EE, early endosomes; Lys, lysosomes; Cyt, cytosol.

The majority of MHC I **(Fig. 6 A)** and MHC II **(Fig. 6 B)** molecules in the endocytic tract accumulated in LEs, in particular in multivesicular late endosomes (MVLE). The remainder were seen in EEs, Lys and small vesicles. Endosomal and lysosomal labeling for MHC I and MHC II was constantly localized on the external membrane and the enclosed membranes. MHC I and MHC II molecules were further detected on

membranes of the endoplasmic reticulum and the Golgi complex, representing the biosynthetic pathway. The cytosol, mitochondria and nuclei showed negligible labeling for both MHC I and MHC II.

To provide evidence for the supposed intersection of MHC I and MHC II pathways in IECs, double-labeling for MHC I and MHC II were carried out on ultrathin sections. Co-localizations were identified throughout the endocytic tract, again predominating in MVLE **(Fig. 6 C)**. As expected, both molecules intensively co-localized at basolateral membranes, while faint co-localization was further seen on microvilli and within the biosynthetic pathway (not shown).

In accordance with the previously described IF experiments, immunogold-labeling confirmed the distinct expression of MHC II in IECs along the crypt-villus axis. Except for the absence of MHC II labeling in crypts of non-inflamed mucosa, the labeling patterns of MHC I and MHC II within IECs did not depend on the maturity of IECs or mucosal inflammation.

To confirm the distinct morphology of MVLE identified on ultrathin sections processed for immunogold-labeling, I analyzed ultrathin sections from ileal mucosa after an alternative embedding technique (resin-embedding). Like in the immunogold experiments, multivesicular endosomes were consistently observed in the supranuclear areas of IECs in all patient groups **(Fig. 6 D)**.

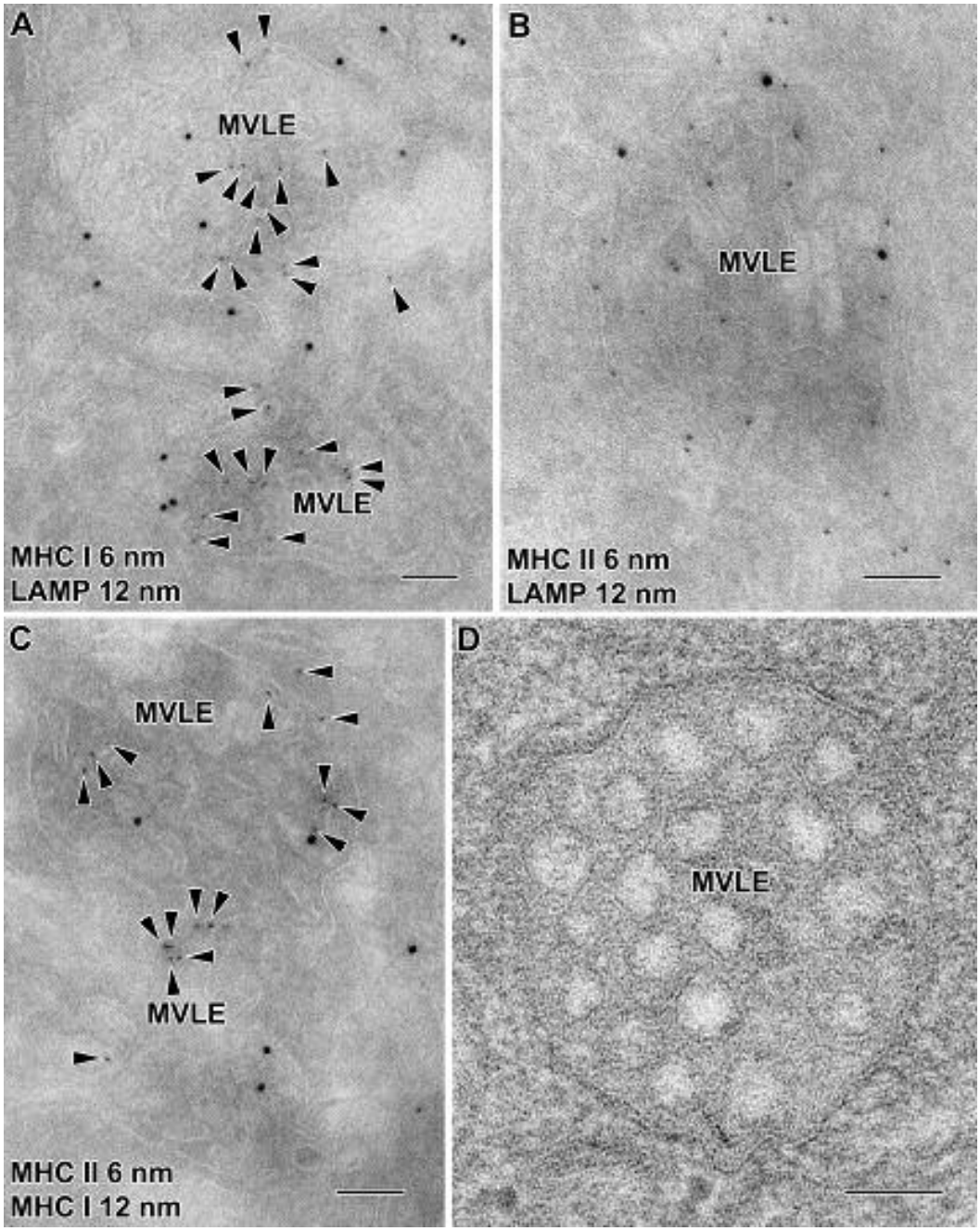


Fig. 6. ***Multivesicular late endosomes are major components of MHC I and MHC II pathways in IECs.*** **(A-C)** Ultrathin cryosections are immuno-labeled for MHC I, MHC II and LAMP-2 with 6nm or 12nm gold particles. Sections of ileal mucosa are prepared from a CD ileitis patient **(A)** and a CD patient in remission **(B, C)**. Multivesicular late endosomes (MVLE), characterized by labeling for LAMP-2, accumulate MHC I **(A)** and MHC II molecules **(B)**. Both molecules are localized on the limiting membranes and internal vesicles of MVLE. Co-localization of MHC I and MHC II is detected in MVLE **(C)** and identifies intersections of the two pathways in these compartments. Of note, images **A** and **C** depict fusion events of MVLE. The double-labeling results showed in the present figure are representative for all patients studied. Image **D** shows the ultrastructural

morphology of a MVLE in IECs after resin-embedding of ileal mucosa (obtained from a CD patient in remission). Bars = 100nm.

Ovalbumin trafficking in the endocytic system of intestinal epithelial cells in Crohn's disease patients and controls

The epithelial transport of the model protein Ag OVA was studied on mucosal biopsies taken from ileal mucosa after *in vivo* incubation with luminally applied OVA. As shortly descibed in the chapter *Materials and methods*, bioptic samples were taken at 0', 5', 10' and 20' after the endoscopical *in vivo* Ag application. The subcellular localization of OVA in the epithelial compartment was analyzed using double-labeling for OVA and LAMP-2 on ultrathin cryosections. Five minutes after exposure **(Fig. 7)**, OVA labeling was detected on microvilli, in engolfements of the apical membrane **(Fig. 7 B)**, in the widened intercellular spaces **(Fig. 7 D)** and also in the LP (the latter, not shown). Regarding the localization in the endocytic system at 5 min time, internalized OVA was almost entirely observed in LAMP-2-negative EEs **(Fig. 7 B)** and various endocytic vesicles situated close to the apical membranes. The majority of MVLE in this period lacked OVA **(Fig. 7 C)**. Lys were also free of OVA. **Fig. 7 A** depicts narrow intercellular cleft apically, (close to the lumen) suggesting an intact physical barrier, with rather modest OVA labeling (tight junctional complex is not catched in this section). The intercellular spaces get widened in the basal pole, showing larger amounts of accumulated OVA clusters **(Fig. 7 D)** on floccular-electrone dense material, more probabely delivered transcellularly and not paracellularly (exosomes could not be described at 5 min period in the intercellular space). The fact that the intercellular space in the proximal (apical site) does held weak labeling, this fact does not exclude the paracellular entrance route for OVA into the intercellular space at the basal/basolateral pole of IECs. This aspect speaks for an inferior paracellular flux, compared to the transcellular flux which implies fast delivery of internalised Ags, especially by EEs into the intercellular space at the basolateral site, confirming other published works on this field. This is an important aspect, since the basolateral site is the major site for MHC-dependent Ag presentation.

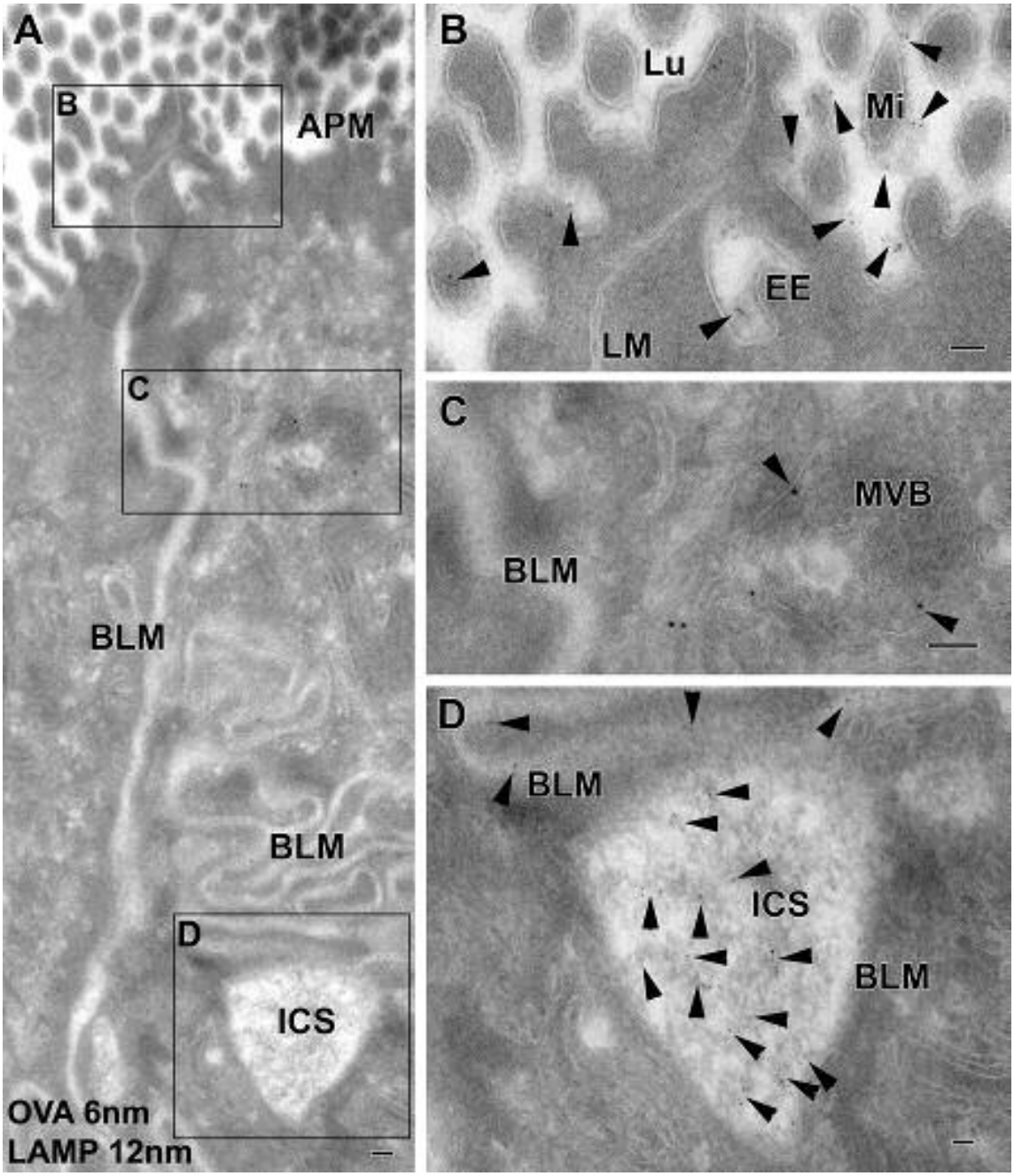


Fig. 7. ***OVA trafficking at 5 min in ileal IECs.*** **(A)** shows an overview of a polarized IEC obtained from a ultrathin cryosections of ileal biopsies of an CD patient in remission; OVA (6 nm) and LAMP-2 (12 nm gold particles). **(B)** The apical membrane (AM) including microvilli (Mi), are the main Ag entry site, label high desity of 6 nm gold particles, representing OVA (pointed by the black arrowheads). In proximity of the AM lies a typical EE, positive for OVA and negative for LAMP labeling. **(C)** depicts an MVLE free of OVA at 5 min timepoint, close to the BLM. Black arrowheads point towards 12 nm particles that stay for LAMP, the typical late endosomal and Lys marker. **(D)** At 5 min, OVA accumulates in the widened ICS (black arrowheads). These results have been observed in all investigated patients groups. Bars = 100nm.

OVA entered LEs and among these MVLE 10 minutes after exposure, as well as equally seen within these compartments after 20 minutes **(Fig. 9 A-C)**.
Lys showed faint labeling for OVA at 10 and 20 minutes **(Fig. 8 A, B)**.

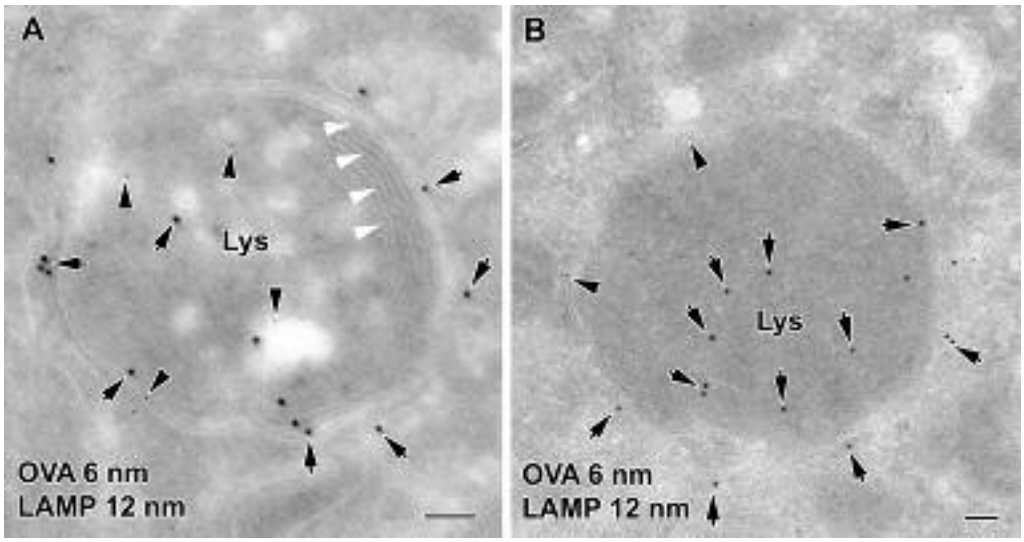


Fig. 8. ***A multilaminar and classical lysosomal compartment in ileal IECs.*** These two micrographs show the typical lysosomal compartments observed on ultrathin cryosections of ileal biopsies obtained from different heathy controls (at 10 min in **A**, and at 5 min), followed by immuno-labeling for OVA (6 nm) and LAMP-2 (12 nm gold particles). **(A)** Multilaminar Lys (or late MIICs) are late endocytic compartments characterized morphologically by an accumulation of internal membrane sheets (white arrowheads). Reminescent vesicles witness the transitional character **(A)**, as hybrid compartments, between classical multivesicular LE and electron-dens (mature) Lys **(** in **B)**. Multilaminar Lys label LAMP-2 (black arrows) on the external limiting membrane sheets and on the internal vesicles. They also containe small amounts of OVA (small gold particles in **A**, featured by black arrowheads) and gradually mature into homogenous electron-dens, round Lys **(B)**. Mature Lys label LAMP-2 (black arrows) both on the limiting membrane and the electrone-dense material, basically absent or faint OVA labeling (black arrowheads) **(B)**. These results have been constatly seen in all investigated patient groups. Bars = 100nm.

Part of the biopsies taken at 20 minutes after *in vivo* exposure were kept in cell culture medium for another 40 minutes at 37°C in order to allow (*ex vivo*) continuity to the endosomal trafficking of the encountered OVA for a period of 60 minutes in total. Sections from biopsies incubated for a 60 minutes period lacked OVA at apical membranes of IECs, instead OVA was found in the intercellular spaces between IECs and within the LP (not shown). The intracellular distribution of OVA in IECs, in

particular regarding LEs/Lys, did not change compared to that observed at 20 minutes period. The time-related subcellular distribution of OVA was similar in crypt and villus IECs and did not differ between CD patients (CD ileitis, CD ileitis in remission) and healthy controls. **Table 1** summarizes traffic routes of OVA in IECs, being representative for all 12 patients exposed to OVA, and analyzing at least 100 IECs per patient. Specimens taken from saline cleaned ileal mucosa before OVA administration (0 minute period) did not show any labeling for OVA (not shown).

	0 min	5 min	10 min	20 min	60 min
APM	-	+	+	+	-
EE	-	+	+	+	- / +
LE	-	- / +	+	+	+
Lys	-	-	+	+	- / +
ICS	-	+	+	+	+

Table 1. ***The dynamics of the subcellular OVA-trafficking in IECs.*** This table illustrates the localization of OVA during trafficking across IECs at different time periods after luminal exposure. The data shown here are representative for all patients studied. APM, apical membrane; EE, early endosome, LE, late endosome; Lys, lysosome; ICS, intercellular spaces.

Targeting of ovalbumin into MHC I-enriched late endosomes of intestinal epithelial cells

"Cross-presentation" of exogenous Ags requires Ag access to MHC I molecules. To study whether and where luminally administered Ags meet the MHC I molecules in the endocytic tract of IECs, double-labelings for OVA and MHC I were carried out on ultrathin sections.

In all patients endoscopically exposed to OVA (CD ileitis, CD ileitis in remission and controls), MHC I pathways intersected the endocytic traffic of OVA in IECs. After 5 minutes exposure, faint OVA labeling was observed in MHC I-positive endosomes, presumably EEs (not shown). In accordance with the subcellular labeling for OVA and MHC I described above, co-localization was consistently found in LEs (especially in MVLE) at later time periods (10, 20 and 60 minutes) **(Fig. 9 D).** Lys revealed faint co-localization for OVA and MHC I at these time points (not shown). Differences between patient groups were not observed.

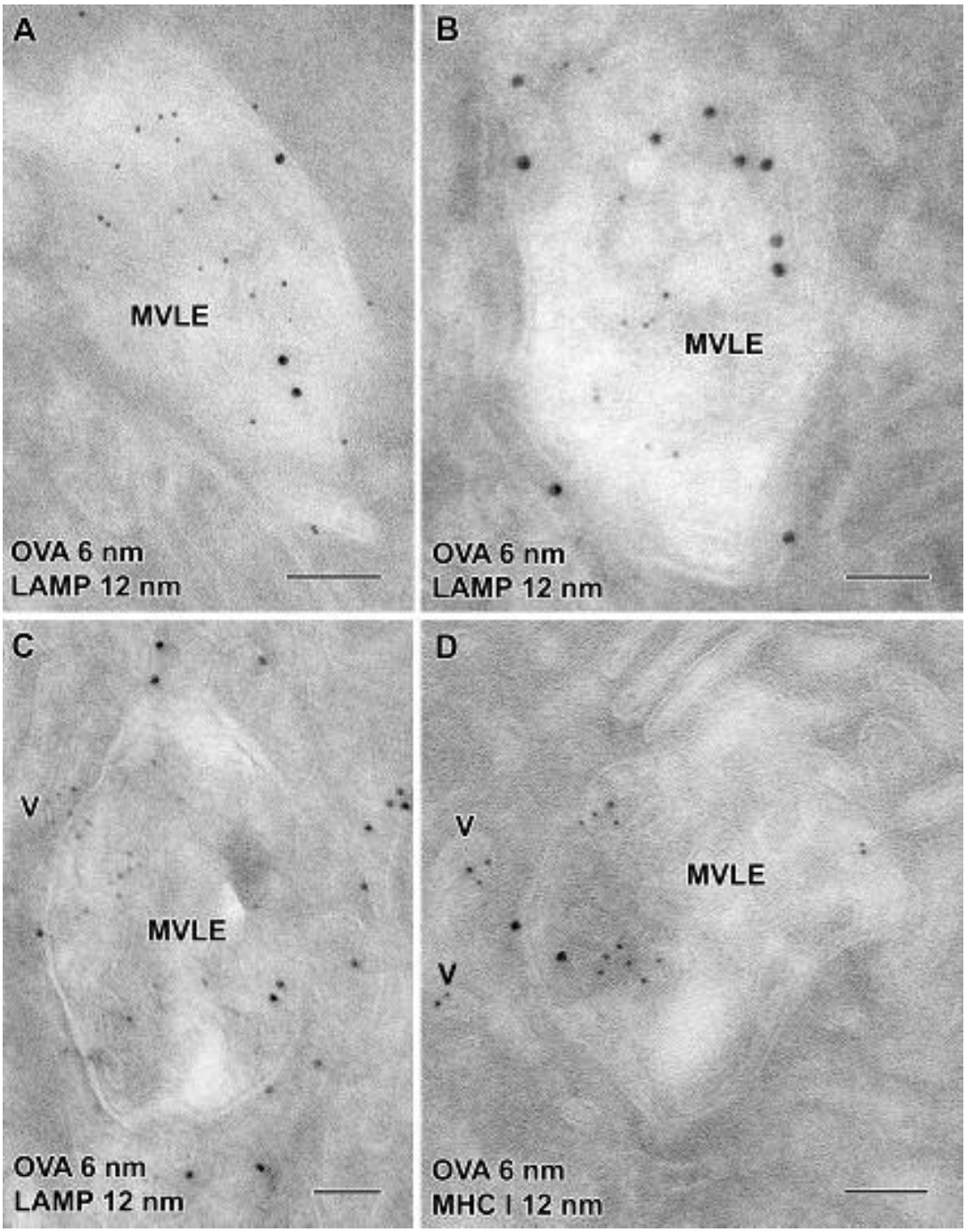


Fig. 9. ***Targeting of luminally administered OVA into multivesicular late endosomes (MVLEs) of IECs.*** Photomicrograph of ultrathin cryosections immuno-labeled for OVA, LAMP-2 and MHC I with 6 nm or 12 nm gold particles. Sections of ileal mucosa are taken from a control **(A, D)**, a CD patient in remission **(B)** and a CD ileitis patient **(C)** 20 minutes after endoscopical exposure **(A, B** and **C)**. At this time, OVA is consistently localized in multivesicular late endosomes (MVLE), labeled for LAMP-2. OVA targeting into MVLE of IECs is not dependent on the inflammatory

state of the mucosa. Image **D** shows co-localization of OVA and MHC I in a MVLE 20 minutes after OVA administration. These results are representative for all patients studied. V, vesicles. Bars = 100 nm.

Figure 10 depicts a detailed representation of the OVA (Ag) trafficking within the complex endosomal system, in human ileal IECs.

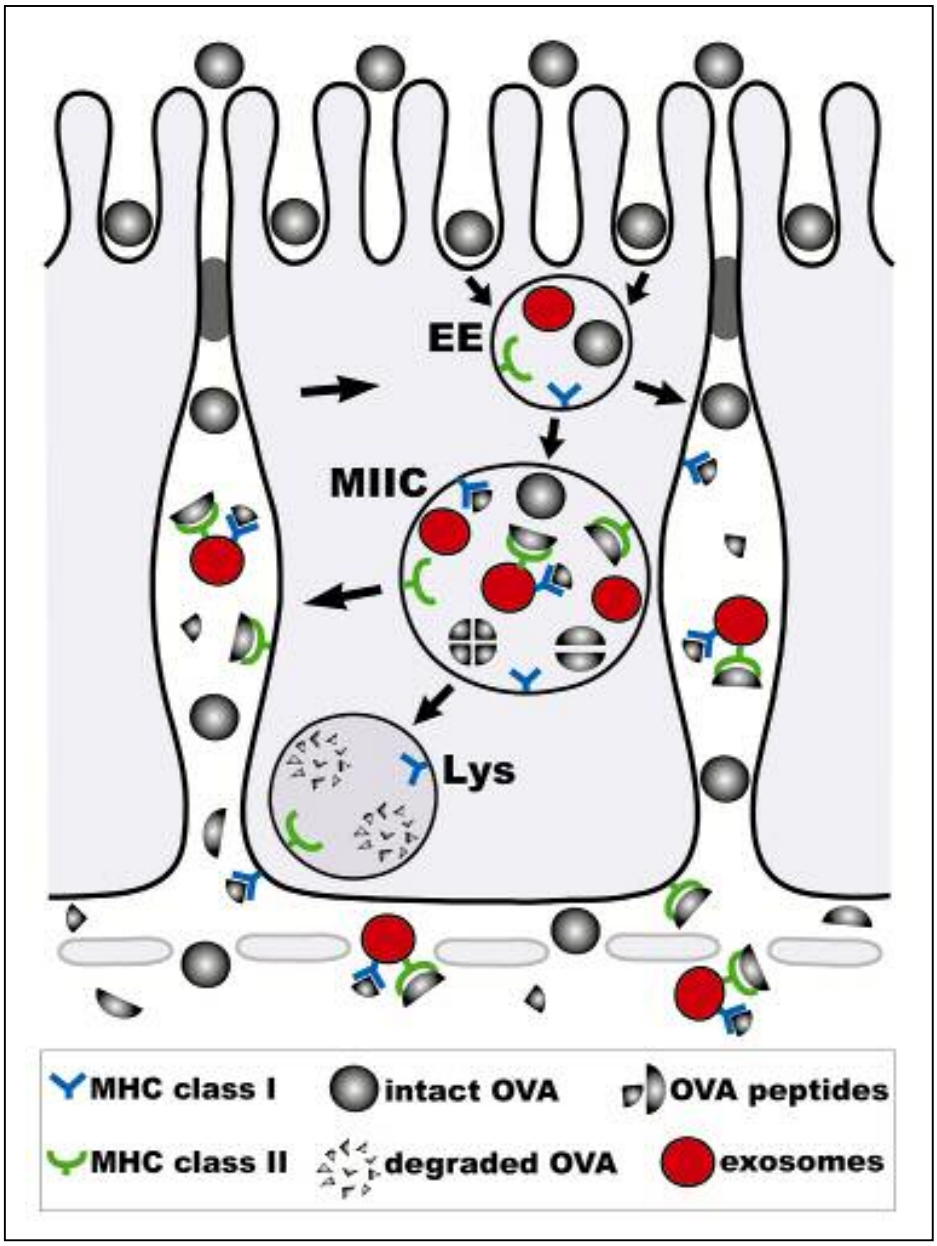


Fig. 10. ***MHC I- and MHC II-restricted presentation pathways of luminal antigens in IECs.*** *(based on the morphological observation made in the present work)*. Undegraded Ags pass through the ileal epithelium transcellularly via EE and / or via paracellular flux. The latter route will most likely be confined to inflamed mucosa. Ags, internalized at apical membranes, will initially enter EEs. Inflammatory stimuli may additionally account for Ag uptake into the endocytic pathway via the

basolateral membranes. Passing EEs, Ags will be delivered into multivesicular late endosomes. Analogous to MHC II-enriched compartments (MIICs) in professional Ag presenting cells, Ags will be processed in these compartments, and their peptides loaded onto MHC I and MHC II molecules. Generated MHC / Ag complexes will subsequently be displayed on the basolateral membranes accessible to adjacent lymphocytes. In addition, MHC : Ag complexes will be released, carried on exosomes, and subsequently exerting systemic immune functions. Part of the internalized Ags will proceed to Lys and undergo complete proteolytic degradation.

Exosomes in the intercellular spaces of the ileal epithelium

Similar to professional APCs (147), recent data suggested the release of MHC I / II-loaded exosomes (30-90 nm in diameter) from IEC lines (59, 77). However, exosomes derived from the epithelial compartment of the gut have not been described *in vivo* yet. To unravel exosomal structures in ileal epithelium, I used an ultrastructural analysis of resin-embedded tissue and found electron-dense content in the widened intercellular spaces, which mainly consisted of amorphous material **(Fig. 11 A)**. In addition, intercellular vesicles (40 to 100 nm in diameter), distinct from basolateral membranes of IECs, were regularly identified within the epithelium **(Fig. 11 A)**. These vesicles were seen independently of mucosal inflammation.

To elucidate MHC protein and LAMP-2 expression on exosomes found in the ileal epithelium, I performed immunogold-labeling experiments. Using double-labeling for MHC I / LAMP-2 and MHC II / LAMP-2, both MHC I **(Fig. 11 B)** and MHC II molecules **(Fig. 11 C)** were detected membrane-bound on extracellular vesicles between IECs. Inflammation did not change the labeling pattern for MHC I and MHC II on the intercellularly situated vesicles. Labeling for LAMP-2 was rarely observed on these structures. As mentioned earlier, OVA was abundantly present within the intercellular spaces of the epithelium already 5 minutes after luminal exposure. Of note, part of this intercellular labeling for OVA was localized on the limiting membranes of intercellular vesicles **(Fig. 112 B)**. While these OVA-labeled vesicles were constantly found 10 to 20 minutes after OVA administration or at later time periods, they were only exceptionally detected after 5 minutes of OVA exposure.

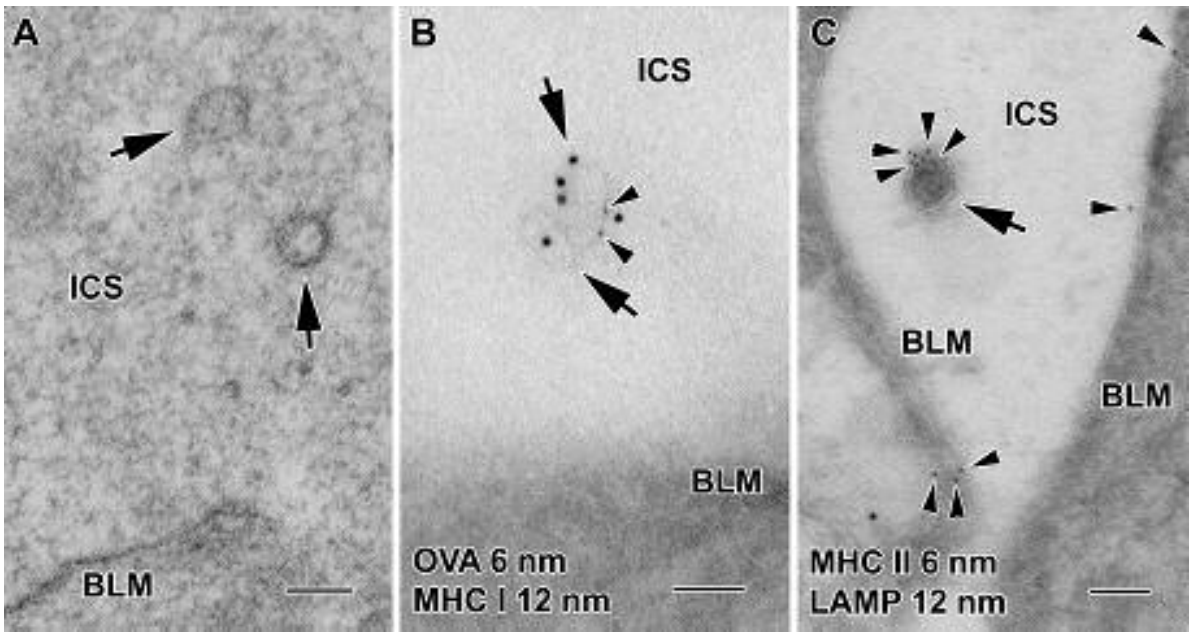


Fig. 11. ***Exosomes bearing OVA, MHC I, and MHC II are identified in the intercellular spaces between IECs.*** Photomicrograph of an ultrathin section made from a resin-embedded specimen **(**A**)** and from biopsies processed for immuno-labeling to visualize OVA (black arrowheads in B), MHC I and MHC II (black arrowheads in **C**) with 6 nm and 12 nm gold particles **(B, C)**. Sections of ileal mucosa were prepared from a CD patient in remission **(A, B)** and a CD ileitis patient **(C)**. (**A**) Exosomes (arrows) are localized in the intercellular spaces (ICS) of the ileal epithelium. Beyond this, the intercellular spaces contain some amorphous electron-dense material. **(B, C)** Exosomes (arrows), identified extracellularly between IECs, carry OVA (20 minutes after exposure), MHC I **(B)** and MHC II molecules **(C)**, but not LAMP-2. BLM, basolateral membrane. These results are representative for all patients studied. Bars = 100 nm.

Discussion

In accordance with previous data also from our group on the epithelial passage of OVA in jejunal and colonic mucosa (98, 100, 142), the terminal ileum shows rapid trafficking processes. Epithelial cross-passage of OVA occurs within 5 minutes after endoscopic exposure. At this time period (5 min), independent of the inflammatory status of the mucosa, OVA is almost entirely restricted to LAMP-2-negative tubulo-vesicular structures in the vicinity of the apical membrane of IECs, meaning EEs and apical endocytotic vesicles. Regarding the fast transepithelial passage, evidence was brought also for other unmodified peptides or proteins (e.g. horsereadish peroxidase) (148). A transcellular route of OVA within this time period probably involves EEs (without degradative properties) and facilitates transepithelial trafficking of intact proteins or peptides exposed to the luminal surface, as also observed by other studies (148, 149). A certain paracellular flow of OVA might also account for a rapid epithelial translocation, though it preferentially occurrs in inflamed mucosa, where tight junctional complexes seem to be damaged (149, 150). Although the junctional complexes appeared well preserved, our experiments within this work definitely exclude a paracellular flux.

Access of internalized Ags to MVLE/MIICs is a prerequisite for processing and binding to either MHC I or MHC II within these compartments (38). In former studies, our group has characterized the intracellular trafficking of luminally given Ags (OVA, gliadin) in epithelial cells of uninflamed mucosa of the duodenum, the jejunum and colon (98-100, 142). Except the germ free SCID mice, we observed a uniform transport of encountered Ags into epithelial MIICs in both mice and humans. Late endosomal targeting of exogenous Ags has also been described in other polarized epithelial cells, such as MDCK cells, after a 15 minutes pulse (151). Consistent with these previous data, the present study shows that endoscopically administered OVA is targeted into MIICs of ileal IECs within a period of 10 to 20 minutes, independent of additional inflammatory stimuli. In addition, colonic epithelial cells featured late endosomal targeting of OVA under constitutive conditions in our previous endoscopic studies (100).

Because the *in vivo* incubation period of up to 20 min was to short for a complete transfer of OVA to the latest components of the endocytic pathway (especially Lys) and a longer *in vivo* incubation was not possible out of ethical reasons, an alternative

strategy was chosen and I performed additional *ex vivo* incubations. At 60 min time, OVA presence shifted towards the cell interiour and late endocytic compartments. Microvilli and apical membrane were free of OVA, and this fact applied for the majority of EEs. LEs, the intercellular space, and in lower amounts Lys labeled OVA too. From the best of our knowledge there are no comparable *in vivo* data in human IECs describing this process at 60 minutes timepoint. Although our experiments could not reveal which of the pathways (the apical, the basolateral, or both) contribute to the transfer of OVA to MIICs, these compartments might represent the suggested common endosome crucially involved in MHC class II-mediated Ag processing in IECs.

The stimulation of Th 1 $CD4^+$ and cytotoxic $CD8^+$ T cells is of great importance in the pathogenesis of CD and is associated with a break down of mucosal tolerance to non-pathogenic Ags of the intestinal lumen (92, 93, 152, 153). Although the priming of naïve T cells in the gut has been classically attributed to DCs, IECs are suggested to participate as well in the Ag presentation of luminal Ags to primed T cells (154). Dependent on inflammatory stimuli, the MHC II-restricted Ag presentation by IECs is belived to result in a clonal anergy of $CD4^+$ T cells, an activation of $CD4^+$ regulatory T cells or a stimulation of pro-inflammatory $CD4^+$ T cells. While the IECs-mediated activation of $CD8^+$ regulatory T cells was shown to involve the non-classical MHC protein CD1d and gp180, the stimulation of cytotoxic $CD8^+$ T cells is suggested to involve classical MHC I proteins (80, 90, 94-96).

In the present work, I have shown that MHC I is constitutively expressed in the ileal epithelium along the entire crypt-villus axis, while MHC II expression is predominantly restricted to villus IECs. In active CD patients, both MHC I and MHC II are well expressed in villus and crypts. The single difference in CD patients in remission is, as in healthy controls the absence of crypt expression for MHC II. This leads to the conclusion that MHC II expression in crypts is upregulated in active CD inflammation, and is reversed during mucosal healing in remission by the inflammatory mileu. This explanation has been suggested in previous reports, when IFN-γ was shown to upregulate MHC II expression in IECs (89, 155).

The findings regarding the cellular MHC II expression in healthy ileal IECs slightly differ from the MHC expression pattern in uninflamed colon IECs. In the healthy colon, IECs lacked MHC II staining or showed a weak epithelial expression (100). However, when affected by CD inflammation, colonic epithelial cells do also reveal a

strong cell surface and endosomal staining for MHC II (89, 100). We have described recently the cell surface expression of induced MHC class II molecules in colon IECs; in this study, MHC II expression was found mainly at the basolateral membrane and marginally at the apical membrane, as in the present study in the ileum. Ag transport into MIICs was confined to mucosal inflammation in Crohn's colitis due to lack of epithelial class II staining in the healthy colon. Thus, the epithelial uptake of luminal Ags into MIICs strongly differs in the small bowel and the colon regarding the quantity of late endosomal MHC II expression. However, the functional relevance of these differences remains unknown. In the present study, MHC I molecules co-localize with OVA in all components of the endocytic pathway in IECs. The most intensive labeling of both proteins is found in MIICs. Our findings strongly suggest that MHC I and MHC II pathways meet the endocytic routes of internalized Ags in MIICs of ileal IECs.

I was unable to detect inflammation-related changes in the subcellular expression of MHC I and MHC II or the intracellular trafficking of OVA. This might explain the dichotomy in the Ag presenting capacity of IECs. In contrast to previous data on colonic epithelial cells, where MHC II was restricted to MIIC (MVLE) of inflamed mucosa (100). In this study, I could detect MHC II in MVLE of ileal IEC in all patients. Constitutive expression of MHC II in villus IECs was extended to crypts in CD ileitis. Crypt IECs affected by CD inflammation show similar trafficking of OVA and expression of MHC II as compared to villus IECs. As a distinct function of immature IECs in Ag uptake has been already reported, Ag presentation by crypt IECs might specifically contribute to the inflammatory processes in CD ileitis (156). Furthermore, there is suggested that the IECs function as APCs might crucially depend on Ag processing properties and the differential involvement of inflammation-related costimulatory molecules such as B-7 proteins (84, 103, 157). Inflammatory conditions were shown to regulate the activity of proteases involved in the MHC-associated processing of exogenous Ags. Thus, dependent on inflammatory stimuli, Ag processing in the IECs MIICs might create MHC : Ag complexes with different immunogenic properties.

The other signalling pathway, MHC I-dependent, has traditionally been suggested to function in the presentation of endogenous, intracellularly derived Ags. Recent data on professional APCs demonstrated that exogenous Ags are efficiently presented via MHC I as well (37). This cross-presentation of exogenous Ags is supposed to occur by cytosolic access of internalized Ags or Ag processing and loading within the

endocytic tract, in particular in MIICs. Functional in vitro data reported that acidic compartments and the activity of acidic hydrolases are essential for Ag specific stimulation of $CD4^+$ T cells by IECs (84, 97). In analogy to the role of MIICs in professional APCs, the findings presented in this thesis indicate that MIICs might be responsible for MHC II-mediated Ag processing in IECs. Our IF experiments, depicting co-localization of both MHC I and MHC II with LAMP-2, suggest that these molecules are present in late endocytic compartments of IECs. Immunogold double-labelings on ultrathin sections gave us the opportunity to further characterize the expression on a subcellular level and showed that MHC I and MHC II molecules co-localize with LAMP-2 mainly in MVLE/MIICs. These findings are in line with the present knowledge and indicate that intracellular MHC II molecule expression lies in the endocytic tract, especially in early and late endocytic compartments, the majority accumulating in MIICs (38, 43). MHC II-restricted Ag processing and peptide loading of internalized Ags has also been suggested for early endocytic compartments, but these events are considered to be characteristic features of MIICs (38, 43, 158).

As described for professional APCs, I was able to demonstrate in this work that MHC II molecules are present throughout the endocytic tract, including EEs, LEs and Lys. A quantitative analysis showed the majority of intracellular MHC II molecules in MVLE, morphologically similar to MIICs in professional APCs. Of note, it was possible to demonstrate that intracellular MHC I molecules are not restricted to the biosynthetic pathway, and that they additionally localized in all components of the endocytic pathway. Similar to MHC II and in line with the data on professional APCs, most of endosomal MHC I labeling is seen in the MIICs of IECs. Our findings using double-labeling for MHC I and MHC II confirm the assumed intersection of MHC I and MHC II pathways in the endocytic tract of IECs, and, again, the most intensive labeling of both molecules is observed in MIICs.

Although our morphological data do not provide definitive evidence for complex binding of exogenous Ags to MHC I or MHC II, they suggest that MIICs are responsible for MHC I- and MHC II-restricted presentation of exogenous Ags by IECs. Further studies will need to clarify the function of the different MHC I- and MHC II-bearing compartments of IECs in Ag processing and presentation. I have demonstated instead the intersection of MHC I and MHC II pathways in MVLE/MIICs. In the same time, I observed in different labeling intensities simple MHC I or MHC II positive MVLE whitin the IECs. Further studies will be necessary to analyze the exact

biochemical components and explaine the difference between the 3 MVLE entities (only MHC I positive, only MHC II positive and co-localized MHC I/II). The factors that discriminate a certain MHC targeting toward a MVLE should be also addressed in future studies (random or genetical encoded) as well.

Recent *in vitro* data demonstrated the secretion of immunocompetent, MHC I- and MHC II-carrying vesicles by IEC lines, referred to as exosomes (59, 77). Exosomes derived from MIICs of B cells or DCs were shown to be capable of activating $CD4^+$ and cytotoxic $CD8^+$ T cells (51, 57). The definitive origin, function and fate of IECs-released exosomes in CD immunopathogenesis is yet to be established. A direct interaction of exosomal MHC:Ag complexes with mucosal T cells as well as an uptake and processing by DCs of the gut mucosa were discussed. In the present work, it was possible to provide *in vivo* evidence in human biopsies, for the presence of epithelial exosomes (40 to 100 nm in diameter) in the gut. Consistent with exosomes derived from IEC lines, I could identify MHC I and MHC II molecules on their limiting membranes. OVA labelling of exosomes in the intercellular spaces of the epithelium at later time points does not prove the presence of processed and MHC-bound OVA-fragments. Recent works demontrated a definitive IEC-specific marker for IEC-originated exosomes and of basolateral surfaces, in mice (159). Several experiments have been made implying A33, but unfortunately the immunolabeling did not function. Nevertheless, these exosomes derive with high probability from the IECs. These OVA/MHC-bearing vesicles might represent immunocompetent exosomes derived from MIICs of ileal IECs and account for a function of IECs in adaptive immunity. Under healthy conditions, the dissemination of exosomes from IECs might be involved in the homeostatic regulation of the response to luminal Ags as suggested in murine models of oral tolerance (160). However, the immunocompetence of IECs released exosomes during mucosal inflammation remains to be elucidated. Follicular DCs were proposed as physiological targets for exosomes, leading to isotype switching and differentiation of T cells (147).

Further studies will need to address the influence of inflammation on the generation of MHC:Ag complexes within the MIICs of IECs, and the related functional consequences in Ag presentation. My results provide evidence that luminal Ags access MHC I molecules in MIICs of IECs, but functional studies should investigate and describe the impact of a cross-presentation by IECs via MHC I to $CD8^+$ T cells. However, it is tempting to speculate that the MHC I-restricted cross-presentation of

exogenous Ags by IECs might be involved in the stimulation of cytotoxic $CD8^+$ T cells and thus promote the inflammatory processes in CD.

Conclusion

The present study provides *in vivo* documentation of the trafficking routes of luminal Ags in human ileal IECs, which are summarized and graphically depicted in **Fig. 10**. I have described the Ag uptake and transport in the classical organells of the endocytic system of IECs in the ileum of healthy individuals and CD patients with regard to the kinetics. I was able to show *in vivo* that MHC I and MHC II pathways meet in MIICs of ileal IECs, compartments which are efficiently targeted by exogenous Ags (like OVA) and are presumably acting similar to MIICs described in professional APCs.

Our findings are in line with previous observations made in MIICs/MVLE of professional APCs like DCs (32, 63) demontrating first *in vivo* data that MHC I co-localizes with MHC II molecules in MIICs of IECs, irrespective of the inflammatory status.

Moreover, I have described the structural basis for the *in vivo* production of MHC- and OVA-bearing exosomes by ileal IECs irrespectively of the inflammatory status. The importance of this fact relies in the potentially immunomodulatory and therapeutic abbilities ascribed to exosomes, as Ag presenting vesicles, which should be the challange for further research in order to be used for novel unerring therapies in autoimmune disorders.

This work brings valuable *in vivo* data not only for the cell biology of ileal IECs, but also for the suggested function of IECs in Ag processing and presentation. These data point out, beside the conventional presentaton towards $CD4^+$ T cells, to a possible cross-presentation of exogenous Ags to cytotoxic $CD8^+$ T cells in CD. IECs may play, therefore, a role in the regulation of immune responses toward luminal Ags.

Summary

Intestinal epithelial cells (IECs) stimulate pro-inflammatory $CD4^+$ and $CD8^+$ T cells during Crohn's disease (CD), as non-professional Ag presenting cells (APCs). Yet, the underlying regulatory mechanisms that determine the functional outcome of Ag presentation within IECs still remained undefined. Since MHC molecules are the main regulators of MHC-dependent Ag presentation, in the present work I have investigated the epithelial expression of MHC I and MHC II and its interference with endocytic pathways, *in vivo*. During ileoscopy, ovalbumin (OVA) was sprayed onto ileal mucosa of CD patients (ileitis and remission) and controls. The epithelial traffic of OVA and MHC I/II pathways was studied in biopsies using fluorescence light and immunoelectron microscopy. I have shown that MHC I is constitutively expressed in the ileal epithelium along the entire crypt-villus axis, while MHC II expression is predominantly restricted to villus IECs. CD inflammation seems to upregulate epithelial MHC II expression in crypts, which is reversed during mucosal healing in remission. Further, I've found intracellular MHC I and MHC II to accumulate within multivesicular late endosomes (MVLE) of IECs, which were targeted by internalized OVA 10 minutes after endoscopical application. Access of internalized Ags to MVLE (morphologically similar to so-called MIICs in professional APCs) is a prerequisite for processing and binding to either MHC I or MHC II within these compartments. By using double-labeling experiments for MHC I and MHC II, I confirm the assumed intersection of MHC I and MHC II pathways in the endocytic tract of IECs, and again the most intensive labeling of both molecules is observed in MIICs. Exosomes carrying MHC I, MHC II and OVA were detected in intercellular spaces of the epithelium. OVA trafficking and labeling patterns for MHC I and MHC II in IECs showed no differences between CD patients and controls. MVLE seem to be responsible for MHC I- and MHC II-related processing of exogenous Ags in IECs and the source of exosomes. Presentation of exogenous Ags by IECs is most likely not restricted to MHC II, but also occurs as "cross-presentation" via MHC I. The distinct Ag presenting capacity of IECs might depend on differential Ag processing within MVLE.

Zusammenfassung

In der Pathogenese des Morbus Crohn (MC) wird angenommen, dass Enterozyten als nicht-profesionelle antigenpräsentierende Zellen in der Lage sind, pro-inflammatorische $CD4^+$ and $CD8^+$ T Zellen zu stimulieren. Die zugrundeliegenden Vorgänge innerhalb der Enterozyten sind noch unzureichend verstanden. Meine Zielsetzung war, erstmals die intrazellulären Transportprozesse luminaler Antigene *in vivo* zu untersuchen. Angesicht der grundlegenden regulatorischen Funktion in der Antigenpräsentierung sollte auch die MHC I/MHC II Expression in Dünndarmenterozyten des terminalen Ileums dargestellt und charakterisiert werden. Darüber hinaus sollten die für die Antigenprozessierung relevanten subzellulären Kompartimente identifiziert werden, um mögliche entzündungsbezogene Unterschiede in der Antigenpräsentierungskapazität der Enterozyten zu identifizieren. Um die vorgenommenen *in vivo* Daten zu erhalten, wurde Ovalbumin (OVA) endoskopisch direkt auf die Ileumschleimhaut appliziert, bei MC Patienten mit aktiver Ileitis oder in Remission sowie an Kontrollprobanden. OVA wurde dabei als prototypisches lösliches Protein benutzt. Anschliessend wurden zu verschiedenen Zeitpunkten mukosale Biopsien entnommen und entsprechend für eine konfokal- und elektronmikroskopische Untersuchung bearbeitet. MHC I und MHC II Moleküle akkumulierten konsistent in multivesikulären späten Endosomen von Enterozyten. Es konnte dabei der Nachweis erbracht werden, dass diese multivesikulären Endosomen wirksam von OVA erreicht werden. OVA-Transport sowie MHC I und MHC II Expressionsmuster waren ohne wesentliche Unterschiede bei MC und Kontroprobanden. Es konnte zusätzlich erstmalig *in vivo* der Nachweis für MHC I/II- sowie OVA-positive Antigen-präsentierende Vesikel, sogenannte “Exosome“, im Interzellularraum, d.h. zwischen den Enterozyten, erbracht werden. Multivesikuläre späte Endosome scheinen - ähnlich wie in professionellen antigenpräsentierenden Zellen - für die MHC I/II-assoziierte Antigenprozessierung und –präsentierung exogener Antigene verantwortlich zu sein, sowie für die Sezernierung der Exosome. Zusätzlich wurde unabhängig vom Patientenkollektiv/Entzündungsstatus der untersuchten Schleimhaubiopsien eine Kolokalisierung des OVA mit MHC I Moleküle in multivesikuläre Endosomen der Enterozyten, als möglicher Hinweise für eine potentielle sogenannte “cross-presentation“ beobachtet. Im Rahmen der “cross-presentation“ werden exogene Antigene nicht nur via MHC-II Transportwege,

sondern auch via eines MHC I Transportweges präsentiert- eine Eigenschaft der professionellen antigenpräsentierenden Zellen.
Die verschiedenen antigenpräsentierenden Fähigkeiten der Enterozyten könnten durch unterschiedliche Antigenprozessierung innerhalb der multivesikulären Endosomen abhängig sein.

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