Modulation der immunologischen Funktionen dendritischer Zellen durch *Yersinia enterocolitica*

Modulation of the immunological properties of dendritic cells by *Yersinia enterocolitica*

DISSERTATION

der Fakultät für Chemie und Pharmazie
der Eberhard-Karls-Universität Tübingen

zur Erlangung des Grades eines Doktors
der Naturwissenschaften

2005

vorgelegt von

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Tag der mündlichen Prüfung:	07.10.2005
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1 Introduction

1.1 Dendritic cells

1.1.1 The role of dendritic cells in the immune system

Host defence against microbial pathogens relies on a concerted action of both antigen-non-specific innate immunity and antigen-specific adaptive immunity (64,84,125). Key features of the innate immune system include the ability to rapidly recognize pathogens and/or tissue injury and the ability to signal the presence of danger to cells of the adaptive immune system (121). The innate immune system comprises phagocytic cells, natural killer (NK) cells, complement and interferons (IFN). Cells of the innate immune system use a variety of receptors to recognize patterns shared between pathogens, e.g. bacterial LPS, carbohydrates, and double-stranded viral RNA (2,32,134). Evolutionary pressure has led to the development of adaptive immunity, the key features of which are the ability to rearrange genes of the immunoglobulin family, permitting creation of a large diversity of antigen-specific clones and immunological memory. However, this highly sophisticated and potent system needs to be instructed and regulated by antigen presenting cells (APCs). DCs are unique APCs because they are the only ones that are able to induce significant primary immune responses, thus permitting establishment of immunological memory (18,20,78,198).

DCs represent a heterogeneous cell population, residing in most peripheral tissues, particularly at sites of interface with the environment (skin, mucosa) (17). They display a high phagocytic capacity. Following tissue damage, DCs process the captured antigens, load them onto MHC molecules and migrate to the secondary lymphoid organs, where they present the antigenic peptides in the context of MHC molecules to T cells, thereby initiating adaptive immune responses (73,205). DCs activate antigen-specific $CD4^+$ T helper cells, which in turn regulate the immune effectors, including antigen-specific $CD8^+$ cytotoxic T cells (CTL) and B cells, as well as non-antigen-specific macrophages, eosinophils and NK cells. Moreover, DCs induce effector cells to home to the site of infection (17). Four stages of DC development have been delineated: (i) bone marrow progenitors; (ii) precursor DCs that are patrolling through blood and lymphatics as well as lymphoid tissues, and that upon pathogen recognition, release large amounts of cytokines, e.g. IFN-γ; (iii)

tissue-residing immature DCs, which possess high endocytic and phagocytic capacity permitting antigen capture; and (iv) mature DCs, present within secondary lymphoid organs, that express high levels of costimulatory molecules permitting antigen presentation and T cell activation (Fig.1.1).

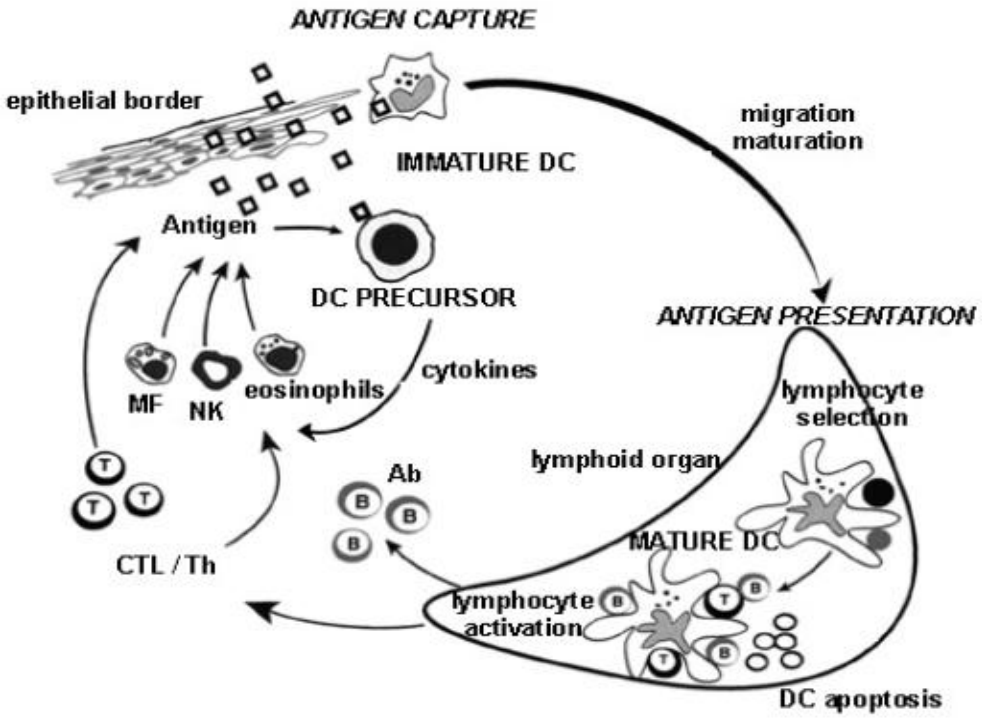

Fig. 1.1: The life cycle of dendritic cells (from Banchereau (17)). Circulating precursor DCs enter tissues as immature DCs. They may directly encounter pathogens that induce secretion of cytokines (e.g. IFN-γ). Immature DCs reside at strategically important sites in the periphery to encounter pathogens. After antigen capture, immature DCs migrate to lymphoid organs where, after maturation, they display peptide-MHC complexes, which allows selection of rare circulating antigen-specific lymphocytes. These activated T cells help DCs in terminal maturation, which allows lymphocyte expansion and differentiation. Activated T lymphocytes migrate and reach the injured tissues. Helper T cells secrete cytokines, which permit activation of macrophages (MF), NK cells and eosinophils. Cytotoxic T cells eventually lyse the infected cells. B cells become activated after contact with T cells and DCs and then mature into antibody-producing plasma cells. It is believed that, after interaction with lymphocytes, DCs undergo apoptosis.

In addition to T cell priming, DCs appear to maintain essentially survival of naive $CD4^+$ T cells (33) and immune T cell memory (159). Importantly, DCs are also involved in the tolerization of the T cell repertoire to self-antigen. This occurs in the thymus (central tolerance) by deletion of developing T cells recognising self-antigens (33), and in lymphoid organs (peripheral tolerance) probably by the induction of anergy or deletion of mature T cells.

1.1.2 Dendritic cell activation and maturation

Immature DCs reside in peripheral tissues at sentinel positions where they take up self and non-self antigens. However, they are not able to present the antigens. Immature DCs accumulate MHC molecules intracellularly and present only a small fraction at the cell surface (37,155). Three types of antigen uptake are known: macropinocytosis, phagocytosis and clathrin-mediated endocytosis (69,172,220,221). Different types of antigens are internalised via these different routes. The constitutive and cytoskeleton-dependent process of macropinocytosis allows rapid and non-specific sampling of large amounts of surrounding fluid and results in the formation of large intracellular vacuoles. Phagocytosis is a receptor-mediated process dependent on actin assembly. In general, the receptors mediating phagocytosis of pathogens are also engaged in clathrin-mediated endocytosis of soluble antigens. The latter allows uptake of macromolecules through specialized regions of the plasma membrane, termed coated pits (194). A large number of endocytic receptors are expressed on immature DCs, namely C-type lectins (93,172,210), receptors for the Fc portion of immunoglobulins (FcRs) (122,152), complement receptors (154), receptors for heat shock proteins (8,21), and scavenger receptors (148).

A signal from pathogens, often referred to as danger signal (67), induces DCs to enter a developmental program, called maturation, which transforms DCs from sentinels into efficient APCs and T cell stimulators (17). The danger signal can be a bacterial or viral product as well as an inflammatory cytokine. Danger signals are recognized through specific pattern-recognition receptors, such as Toll-like receptors, FcR and cytokine receptors (125,152).

Maturation of DCs is accompanied by fundamental morphological and functional changes. Antigen uptake, phagocytosis as well as macropinocytosis is down-regulated (172). In addition, MHC molecules are redistributed from intracellular endocytic compartments to the cell surface (37,155). Antigen processing, peptide loading as well as the half-life of MHC molecules is increased (37,155). Finally, the surface expression of T cell co-stimulatory molecules, such as CD80, CD86 or CD40, also rises (Fig. 1.2).

Simultaneously with the modifications of their antigen presentation abilities, maturation induces migration of DCs out of peripheral tissues (9) to the secondary

lymphoid organs. Modifications in the expression of chemokine receptors (e.g. CCR7) and adhesion molecules (e.g. ICAM-I), as well as profound changes of the cytoskeleton organization contribute to the migration of DCs (173).

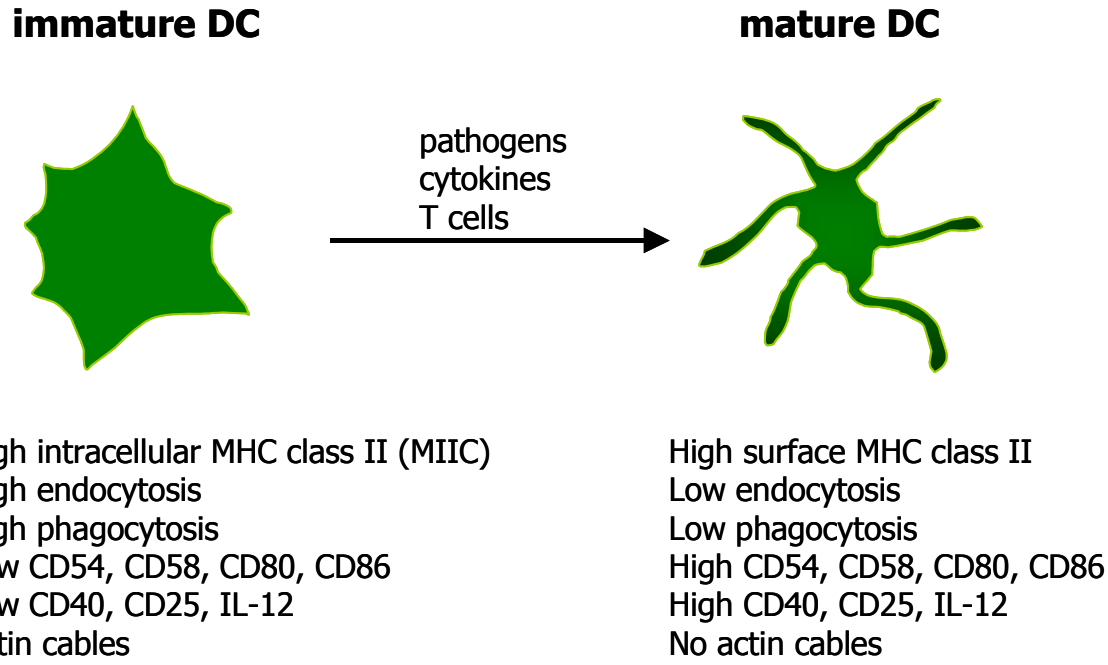

Fig. 1.2: Features that change during DC maturation.

1.1.3 Mouse dendritic cells

At least three distinct pathways of DC development have been identified in mice, the myeloid, the lymphoid and recently the plasmacytoid pathway. Evidence for a myeloid DC lineage derives mainly from *in vitro* differentiation studies, in which myeloid-committed precursors give rise to both granulocytes/monocytes and myeloid DCs under the influence of granulocyte/macrophage colony-stimulating factor (GM-CSF) (88,176). DCs can also arise from lymphoid-committed precursors (5,174,199,217,222). Lymphoid and myeloid DCs differ in phenotype, localization, and function. Both subsets express high levels of CD11c, MHC class II, and the co-stimulatory molecules CD86 and CD40. The former hypothesis that mouse $CD8\alpha^-$ DCs were myeloid-derived and $CD8\alpha^+$ DCs lymphoid-derived has been challenged by recent findings that $CD8\alpha^-$ and $CD8\alpha^+$ DCs could both be derived from either lymphoid- or myeloid-committed precursors (119,149). Markers such as DEC-205 and CD1d are expressed at higher levels on lymphoid DCs, but they can be up regulated

on myeloid DCs by *in vitro* culture (119,149,216,222) or LPS treatment. Lymphoid DCs are localized in the T cell–rich areas of the periarteriolar lymphatic sheaths (PALS) in the spleen and lymph nodes (51,109,149,199). In contrast, myeloid DCs are in the marginal zone of the spleen (51,109,149,199) but migrate to the PALS by signals such as LPS (51). The lymphoid DCs produce higher levels of interleukin (IL)-12 (118,138,149,153) and are less phagocytic than myeloid DCs (109,149). *In vitro*, the lymphoid DCs were reported to prime allogeneic CD4 and CD8 T cells less efficiently than myeloid DCs (100,201). *In vivo*, both lymphoid and myeloid DCs appear to prime antigen-specific $CD4^+$ T cells efficiently (118,150). The mouse counterpart for human plasmacytoid DCs shows an immature $CD11c^{low}$, $CD8\alpha^-$, $CD11b^-$, $CD45RA^+$ phenotype and produce IFN-α.

1.2 Antigen processing and presentation

$CD8^+$ and $CD4^+$ T cells express clonally distributed T cell receptors (TCR) which recognize antigenic peptides associated with MHC class I and MHC class II molecules, respectively. A more or less strict compartmentalization of MHC class I and MHC class II biogenesis results in the loading of exogenous antigens on MHC class II molecules in the endocytic pathway and the selective loading of endogenous antigens on MHC class I molecules in the endoplasmic reticulum (ER).

1.2.1 MHC class I restricted antigen presentation

To generate $CD8^+$ cytotoxic killer cells, APCs present antigenic peptides on MHC class I molecules. Most of these MHC class I ligands are derived from endogenous proteins (Fig. 1.3). These cytosolic proteins are degraded by the proteasome (38), further trimmed by cytosolic peptidases, and the derived peptides are then translocated into the ER via ATP-dependent TAP1/2 transporters (189). The TAP transporters belong to the family of ABC (ATP-Binding-Cassette) transporters. The length of the peptides varies between 8-14 amino acids (208). The loading of peptides onto newly synthesized MHC class I molecules takes place within the ER. The MHC class I-peptide complexes are then transported through the Golgi apparatus to the cell surface. Several chaperones are needed for the transport of the peptides from the proteasome to the TAP and from the TAP to the MHC class I molecule (7,105,106,197).

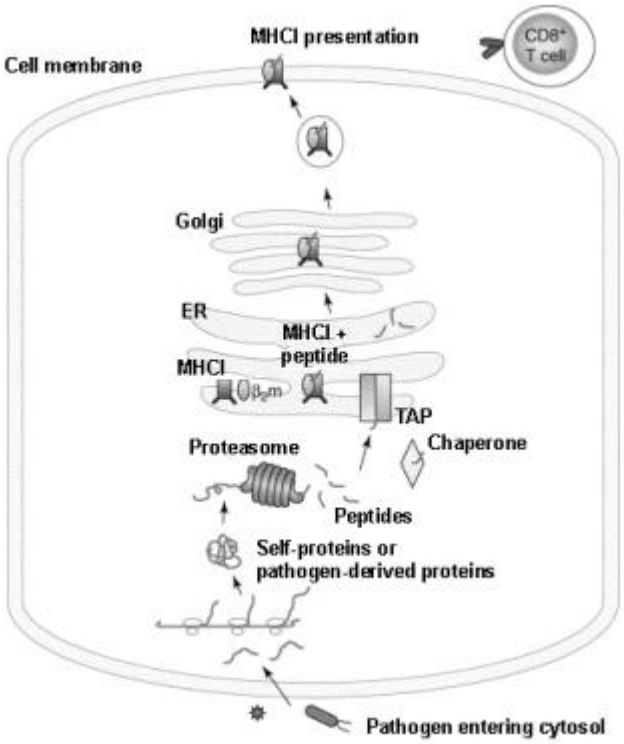

Fig. 1.3: MHC class I restricted antigen presentation (from Salome Landmann (107)).

1.2.2 MHC class II restricted antigen presentation

To activate CD4 T cells the antigenic peptide has to be presented on MHC class II molecules on the surface of APCs. The processing of exogenous antigens for presentation on MHC class II molecules occurs within the endocytic pathway (Fig. 1.4). This comprises the uptake of exogenous proteins by phagocytosis, macropinocytosis or clathrin-mediated endocytosis. The resulting vesicles fuse with lysosomes in which degradation of the proteins is mediated by cathepsins and proteases.

MHC class II molecules are synthesized in the ER. Soon after synthesis three α/β MHC class II dimers associate with a trimer of invariant chains (Ii) (49). The nonamers exit the ER and pass through the Golgi apparatus to a specific endocytic compartment, called MHC class II compartment (MIIC), in the late endosomal pathway. In the MIIC the Ii is degraded by several proteolytic enzymes of the cathepsin family (212).

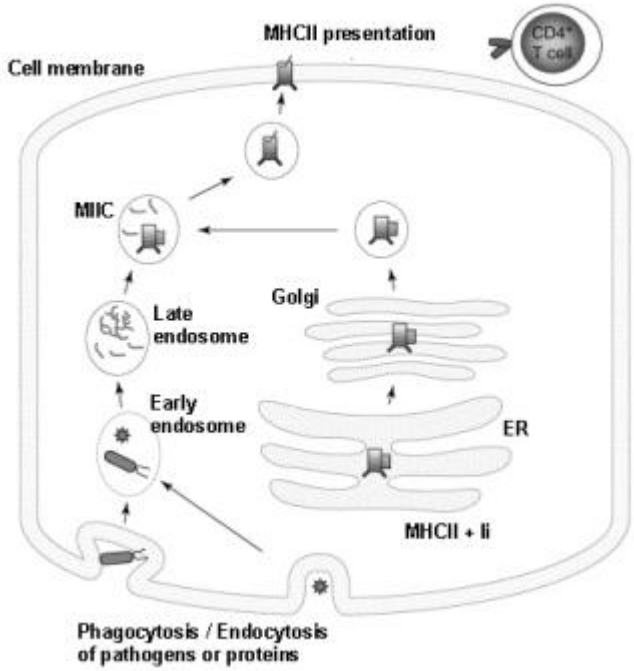

Fig. 1.4: MHC class II restricted antigen presentation (from Salome Landmann (107)).

A central region of the Ii, termed class II-associated invariant chain peptide (CLIP), occupies the peptide-binding groove of class II molecules (158,186) and prevents peptide binding of the MHC class II molecules in the ER and during the transport to the MIIC compartment (204). MHC class II dimers bind antigenic peptides under the control of two nonpolymorphic MHC class II molecules HLA-DM/H2-M and HLA-DO/H2-O (in human/mouse) (101). These two molecules promote catalytic removal of CLIP and enhance peptide binding to MHC class II molecules (35,49). Once loaded with peptides, MHC class II molecules are transported to the plasma membrane.

1.2.3 T cell activation

The crucial first step in adaptive immunity is the activation of naive antigen-specific T cells by professional antigen presenting cells (APCs). APCs display three types of protein molecules on their surface that contribute to the activation of T cells to become effector T cells: (1) MHC molecules, which present foreign antigen to the T cell receptor, (2) co-stimulatory molecules (CD80, CD86, CD40), which bind to complementary receptors (CD28, CD40L) on the T cell surface, and (3) cell-cell adhesion molecules (CD54, CD58), which enable a T cell to bind to the antigen-

presenting cell for long enough to become activated. The activation of T cells by professional APCs leads to their proliferation and the differentiation of their progeny into armed effector T cells. A few of the T cells become memory T cells. The proliferation and differentiation depends on the production of cytokines (e.g. IL-2).
Three types of effector T cells exist. CD8 T cells or cytotoxic T lymphocytes, which recognize pathogen-derived peptides bound to MHC class I molecules (mostly from viruses). CD4 T cells recognise peptides on MHC class II molecules. Depending on the cytokine secretion pattern CD4 T cells develop into subtypes of T-helper cells: T_H1 cells or T_H2 cells.

1.3 Infection and Immunity

As mentioned in Chapter 1.1.1 host defence against pathogens is based on the non-specific or innate immune system and the specific or adaptive immune system. The innate immune system is also the first line of defence against a pathogen because it is permanent present. Components of the non-specific defence are skin, epithelial cells, phagocytic cells, and complement. The second line of defence is specific for a particular type of bacterium or virus and is thus called specific immune system.

1.3.1 Innate immune system

1.3.1.1 Pattern recognition receptors

The innate immune system uses a variety of pattern recognition receptors (PRRs) that can be expressed on the cell surface, in intracellular compartments, or secreted into the bloodstream and tissue fluids (125). The principal function of PRRs is to detect a limited set of conserved molecular patterns (pathogen-associated molecular patterns (PAMPs)) that are unique to microbes and to signal the host the presence of infection. The PRRs include the acute phase proteins mannan-binding lectins, C-reactive protein, and serum amyloid protein as secreted pattern recognition molecules. The macrophage mannose receptor, the macrophage scavenger receptor and MARCO belong to the PRRs expressed on the cell surface.
The Toll-like receptor (TLR) family is the best-characterized class of PRRs in mammalian cells. TLRs detect multiple PAMPs (202), including lipopolysaccharide (LPS) (detected by TLR4), bacterial lipoproteins and lipoteichoic acids (detected by TLR2), flagellin (detected by TLR5), the unmethylated CpG DNA of bacteria and

viruses (detected by TLR9), double stranded RNA (detected by TLR3) and single-stranded viral RNA (detected by TLR7) (80). TLRs 1, 2, 4, 5, 6 are expressed on the cell surface and are specialised to recognize mainly bacterial products. In contrast TLRs 3, 7, 8 and 9 are localized in intracellular compartments (80,120) and detect viral nucleic acids in late endosomes and lysosomes (58,113).
All PRRs sense microbial infection and trigger a multitude of antimicrobial and inflammatory responses like opsonization, activation of complement, phagocytosis, activation of proinflammatory signalling pathways, and induction of apoptosis.

1.3.1.2 Complement

The complement system is a set of plasma proteins that act together to attack extracellular pathogens. Complement can be activated spontaneously by certain pathogens or by antibody binding to the pathogen. The pathogen becomes coated with complement proteins that facilitate pathogen removal by phagocytes and can also kill the pathogen directly (92).

1.3.1.3 Phagocytes

There are two major groups of phagocytes, macrophages and polymorphonuclear leukocytes (PMNs). Phagocytic cells play a key role in all phases of host defence. In addition to engulfing opsonized particles coated with antibodies and/or complement, they can recognize and ingest many pathogens directly. Indeed, the same complement receptors by which they engulf opsonized particles recognize various microbial constituents. Leukocyte integrins CD11b/CD18 (CR3) and CD11c/CD18 (CR4), which are expressed particularly on macrophages, monocytes and PMNs, recognize the inactivated form of the complement component C3b that remain attached to the pathogen surface and stimulate phagocytosis of the pathogens.
When pathogens cross an epithelial barrier, they are immediately recognized by phagocytes in the subepithelial connective tissues leading to trapping, engulfment, and destruction of the pathogen. In addition to phagocytosis, interaction of pathogens with phagocytes leads to secretion of cytokines (92).

1.3.1.4 Inflammatory cytokines

One important function of the innate immune response is the recruitment of phagocytic cells and thereby effector molecules to the site of infection by the release of a number of cytokines and other inflammatory mediators. The cytokines secreted by phagocytes are a structurally diverse group of molecules including interleukin-1 (IL-1), IL-6, IL-8, IL-12, and tumor necrosis factor-α (TNF-α). IL-1 activates vascular endothelium and lymphocytes. IL-6 induces fever and the production of acute-phase proteins (serum amyloid protein, C-reactive protein, fibrinogen, mannan-binding lectin) in the liver. IL-8 is a CXC chemokine involved in neutrophil recruitment and activation. IL-12 activates NK cells and induces the differentiation of CD4 T cells into T_H1 cells. TNF-α activates vascular endothelium and increases vascular permeability, which leads to increased entry of IgG, complement, and cells to tissues and increased fluid drainage to lymph nodes.

Cells infected with viruses produce interferons. These slow down viral replication and enhance the presentation of viral peptides to cytotoxic T cells as well as activating NK cells, which can distinguish between infected and uninfected host cells.

1.3.2 Adaptive immune system

Although the non-specific innate defences are effective, they do not protect against all invading pathogens. Microbes have developed several strategies to evade the innate immune system. Therefore a second defence system has evolved, the adaptive immune system, including antibodies and T cells.

1.3.2.1 Antibodies

Antibodies are protein complexes produced by B lymphocytes (B cells). There are several types of antibodies with different immunological functions. During infection IgM and IgG1-4 are most important. IgM antibodies are synthesized in the early phase of the immune response and are found mainly in the blood. They are pentameric in structure and specialised to activate complement efficiently upon binding antigen. IgG1 and IgG3 are called opsonizing antibodies because these two subtypes are the most effective in opsonizing microbes. This opsonization facilitates the ingestion of antigens by phagocytes. IgG also mediates the attachment of cytotoxic cells to infected tissue, eventually leading to death of an infected cell

(antibody-dependent cell-mediated cytotoxicity). This mechanism serves as a defence against intracellular pathogens. Both IgG and IgM neutralize microbes by binding to the surfaces of bacteria and viruses and prevent them from attaching to and invading host cells. Both types of antibodies are also able to activate complement and neutralize toxins (92).

1.3.2.2 Cytotoxic T lymphocytes (CTLs)

On their surface, CTLs express a protein called CD8, which is associated with the T cell receptor, by which they recognize pathogen-derived peptides bound to MHC class I molecules (mostly from viruses). CTLs play a role in killing infected host cells similar to that of NK cells and produce proteolytic enzymes (granzymes) that trigger apoptosis in the infected cells. This type of attack kills the infected cells but commonly not the microbes. Microbes, released from infected cells, can be taken up by activated macrophages, which finally kill them. Alternatively, the CTLs might also attack the microbes by perforin and granulysin stored within their granules. First, perforin forms pores into the host cell membrane. By these pores granulysin enters the infected cell and kills the bacteria by creating pores in their membranes.

1.3.2.3 T helper cells

After activation naive CD4 T cells differentiate into either T_H1 cells or T_H2 cells. IL-12 produced by PMNs or DCs stimulates NK cells to produce IFN-γ, which in turn stimulates CD4 T cells to differentiate into T_H1 cells. IL-4 stimulates $CD4^+$ T cells to develop into T_H2 cells. T_H1 cells produce IFN-γ, which activates macrophages allowing them to destroy intracellular microorganisms more efficiently. T_H1 cells can also stimulate B cells to produce subclasses of antibodies and activate cytotoxic T cells. T_H2 cells activate eosinophils and stimulate B cells to produce antibodies of the IgG1 class by secreting IL-4 and IL-5.

1.3.3 Host defence against intracellular and extracellular bacteria

Infectious agents can grow in various host compartments divided into extracellular compartments like intestinal lumen, tissue and blood, and intracellular compartments including cytoplasma and vesicles. Extracellular bacteria are usually susceptible to killing by phagocytes and thus have developed means to resist engulfment like

capsules and toxins. Antibodies as well as the complement system also provide protective immunity against extracellular bacteria. Intracellular pathogens invade host cells in order to replicate either in the cytosol (*Chlamydia* ssp. and *Listeria monocytogenes*) or in vesicles (*Salmonella typhimurium* and *Legionella pneumophila*). Virulence factors called invasins can trigger uptake of bacteria in non-phagocytic cells. One strategy of surviving phagocytosis is to escape the phagosome before it fuses with lysosomes. Alternatively, some bacteria are able to prevent phagolysosomal fusion by preventing acidification of the vacuole.

1.4 The genus *Yersinia*

The genus *Yersinia* belongs to the family of *Enterobacteriaceae*. They are facultative anaerobe, gram-negative rods with an optimal growth at 27-30°C (45). The genus *Yersinia* includes three species that are pathogenic for rodents and humans: *Y. pestis* is the causative agent of plague, *Y. pseudotuberculosis*, and *Y. enterocolitica* cause acute or chronic enteric disorders such as enteritis, enterocolitis, and mesenteric lymphadenitis, which are normally self-limited. In some cases the infection leads to systemic manifestations like septicaemia and immunopathological sequelae including reactive arthritis, uveitis, and erythema nodosum (48). While a fleabite generally inoculates *Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica* are food-borne pathogens. In spite of these differences in infection routes, all three have a common tropism for lymphoid tissues and a common capacity to resist non-specific immune response, in particular phagocytosis and killing by macrophages and PMNs. Electron microscopy as well as cell biology examinations revealed that *Y. enterocolitica* and *Y. pseudotuberculosis* are largely located extracellularly (12,55,77,188,191).

1.4.1 Pathogenicity factors of *Y. enterocolitica*

Yersinia possesses several virulence factors encoded either by genes of the bacterial chromosome (157) or by genes located on the 70-kb virulence plasmid pYV (*plasmid Yersinia Virulence*) (47,87,145,195) which enables *Yersinia* spp. to survive and multiply in the lymphoid tissues of their host. Table 1 (pages 19 and 20) gives an overview of the pathogenicity factors of *Yersinia spp.*

Table 1: Overview of the most important virulence factors of enteropathogenic *Yersinia*

a) Chromosomally-encoded factors

Factor	Function	Literature
Ail	Mediates adhesion to and invasion in epithelial cells as well as serum resistance.	(25,129,147)
Inv	Invasin, an adhesin. Initiates by binding to β1-integrins transport of *Yersinia* across the M cells, internalisation in epithelial cells and secretion of IL-8.	(6,31,60,91,95,114,144,181,184)
Myf	Fibrillae possibly acts as a colonization factor and leads together with Yst to diarrhea.	(90)
SodA	Super oxide dismutate (Sod), enzyme for detoxification of exogenous oxygen radicals produced by phagocytes. Mediates virulence for colonization of liver and spleen, but not of Peyer's patches.	(161)
Yst	Heat-stable enterotoxin, stimulates the activity of guanylate cyclase of intestinal epithelial cells and leads to diarrhea.	(52,151,160)
HPI (High Patho-genicity Island)	The HPI encodes the extracellular siderophore Yersiniabactin (Ybt) that enables the bacteria to multiply under iron-depleted conditions. Genes involved in Ybt synthesis, transport and regulation are clustered in the HPI. Ybt possibly acts immunosuppressive on T and B cells, macrophages and PMNs.	(11,15,75)

b) Plasmid-encoded factors

Factor	Function	Literature
YopE	Cytotoxin, works as GAP (GTPase activating protein) for Rho-family proteins, blocks phagocytosis, disrupts actin filaments.	(1,23,165,215)
YopH	Phosphotyrosine phosphatase, which dephosphorylates proteins of focal adhesions therefore responsible for up to 50% of the antiphagocytic activity of yersiniae towards neutrophiles and macrophages. Suppression of oxidative burst in macrophages, inhibition of T and B lymphocyte activation.	(22,24,72,170,175,223)
YopO (YpkA)	Serine-threonine kinase activated by G-actin causing autophosphorylation of serine residues. Disruption of actin filaments. Interacts with RhoA and Rac1.	(19,68,94)
YopM	Strongly acidic protein containing leucine-rich repeats (LRRs). It traffics to the cell's nucleus by means of a vesicle-associated pathway.	(111,112,123,193)
YopP	Cysteine protease, interacts with members of the MAPK kinase (MKK) super family and IKK-β thereby disrupting MAPK and NF-κB signalling pathways leading to inhibited release of proinflammatory cytokines as well as induction of apoptosis in macrophages.	(30,53,54,141,142,167)
YopT	Destruction of actin stress fibres by modification of RhoA, which is released from the plasma membrane and accumulates as monomeric protein in the cytosol.	(89,146,224)
YadA	Mediates adherence to epithelial cells and phagocytes. Binds to collagen, fibronectin, and laminins. It protects *Y. enterocolitica* against killing by PMNs and lysis by complement due to the binding of factor H by YadA.	(40,162,163,185)
LcrV	Suppression of TNF-α and IFN-γ. Inhibits the chemotaxis of neutrophils. Induces the production of IL-10 in macrophages.	(135,192,219)

1.4.2 The type III secretion system of *Y. enterocolitica*

By means of its type III secretion system (TTSS) gram-negative bacteria are able to translocate their effector proteins directly into the cytosol of host cells (66). To date 20 different TTSS are identified in animal and plant pathogens (46). The pYV plasmid encodes the Yop virulon, a system consisting of secreted proteins called Yops and their dedicated type III secretion apparatus called Ysc.

Upon contact with eukaryotic target cells, *Yersinia* bacteria build several syringe-like organelles at their surface (Fig. 1.5). These organelles, called the Ysc injectisome, are protein pumps spanning the peptidoglycan layer and the two bacterial membranes topped by a stiff needle-like structure protruding outside the bacterium. The whole organelle comprises 27 proteins. The internal part contains 10 proteins, which have counterparts in the basal body of the flagellum, indicating that the two organelles have a common evolutionary origin. The external part of the injectisome, which spans the bacterial outer membrane, is a homomultimeric ring-shaped structure with a central pore of ≈50 Å (98). The Ysc injectisome ends with a 60–80 nm long and 6–7 nm wide needle, whereas the length is genetically controlled (130). Effector Yops destined for secretion through the injectisome have no classical signal sequence (128). Nevertheless, a minimum of 15 residues at the NH_2 terminus are necessary for Yop secretion (4,196).

To become secreted some of the Yops need the help of small individual chaperones called Syc proteins. There are two categories of Yop proteins. Some are intracellular effectors, whereas the others are "translocators" which are needed to deliver the effectors across the eukaryotic plasma membrane into the cytosol of eukaryotic cells. The translocators (YopB, YopD, LcrV) form a pore of 16-23 Å in the eukaryotic cell membrane.

Six effector Yops have been characterized: YopE, YopH, YopM, YopJ/P, YopO/YpkA, and YopT. YopH is a powerful phosphotyrosine phosphatase playing an antiphagocytic role by dephosphorylating several focal adhesion proteins. YopE and YopT contribute to antiphagocytic effects by inactivating GTPases controlling cytoskeleton dynamics. YopP plays an anti-inflammatory role by preventing the activation of transcription factor NF-κB. YopP also blocks the MKKs, inhibiting the activation of MAPK, which abrogates activation of CREB, a transcription factor involved in the immune response. YopP induces apoptosis in macrophages, either

directly by acting upstream of Bid or indirectly by blocking the synthesis of NF-κB–dependent antiapoptotic factors. Less is known about the role of the phosphoserine kinase YopO/YpkA and YopM (see also Table1 b, page 20).

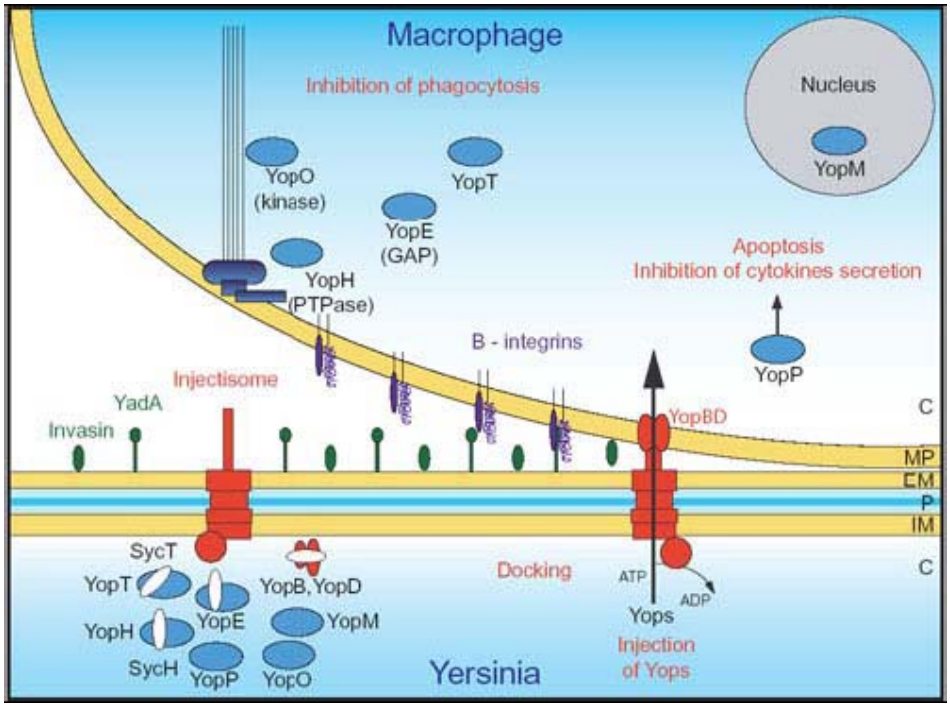

Figure 1.5: **Secretion of Yops by the Ysc injectisome and translocation across the target cell membrane.** (from Cornelis 2002) When *Yersinia* is placed at 37°C in a rich environment, the Ysc injectisome is installed and a stock of Yop proteins is synthesized. During their intrabacterial stage, some Yops are capped with their specific Syc chaperone. Upon contact with a eukaryotic target cell, the adhesins YadA or Inv interact with integrins and the bacterium docks at the cell's surface. Then, the secretion channel opens and Yops are exported. YopB and YopD form a pore in the target cell plasma membrane (MP), and the effector Yops are translocated across this membrane into the eukaryotic cell cytosol (C). EM, outer membrane; P, peptidoglycan; IM, plasma membrane.

1.4.3 Immune response to *Yersinia*

Enteric *Yersinia* spp. are enteroinvasive pathogens, which are taken up by M cells. M cells are specialized epithelial cells which occur within the follicle-associated epithelium of the Peyer's patches (12,71). M cells expose β1-integrins at their apical ("lumenal") surface, which may bind to the *Yersinia* invasin protein (41,182). The entry of enteric *Yersinia* by this route is an ambiguous process: on the one hand, *Yersinia* may use M cells as a structure of the mucosal immune system for invasion of host tissue, and most likely for subsequent dissemination. On the other hand, the mucosal immune system is stimulated upon entry of *Yersinia* at the very site where

efficient immune responses are usually initiated. One day after M cell invasion by *Y. enterocolitica*, small microabscesses consisting of PMNs and extracellularly located *Yersinia* can be detected in Peyer's patch tissue (14,77).

Yersinia has evolved efficient mechanisms mediated by the outer membrane protein YadA and secreted anti-host effector proteins (Yops) (for references see table 1 page 19/20) to evade host innate defence mechanisms including phagocytosis by PMNs, macrophages and the complement system. In accordance with these *in vivo* observations, *Yersinia* manifests some resistance to phagocytosis *in vitro*, both by macrophages (63,164) and by PMN (40,170,213). After replication in Peyer's patches, enteric *Yersinia* disseminate via the lymphatics and possibly via the blood stream to the mesenteric lymph nodes, spleen, liver, lungs and peripheral lymph nodes (12).

The adaptive immune response plays a crucial role in the clearance of a *Yersinia* infection. It has been clearly demonstrated that *Yersinia* infection leads to strong T cell responses, including activation and proliferation of CD4 and CD8 T cells, and that these T cells are involved in control of *Yersinia* (13,16,26,29,61,62,137). In fact, mice deficient for T cells are unable to control the pathogen, and therefore develop chronic progressive and fatal infection (13). Adoptive transfer of *Yersinia*-specific $CD4^+$ or $CD8^+$ T cells consistently mediates resistance to a normally lethal challenge of *Yersinia* (16). As protective CD4 or CD8 T cells produce cytokines such as IFN-γ and IL-2, but not IL-4 or IL-10, it can be concluded that T_H1 or IFN-γ producing cytotoxic T cells are protective in yersiniosis (14,16,26,28). T_H1 cells produce predominantly IFN-γ, and it is established that IFN-γ can activate macrophages which in turn might be able to kill the pathogen.

The role of cytokines in yersiniosis has been extensively studied in mouse strains that are relatively susceptible (e.g., BALB/c) or resistant (e.g., C57BL/6) to *Yersinia* infection. One reason for this differential susceptibility of mice is their different ability to mount T_H1 responses and produce IFNγ upon *Yersinia* infection (10,27). *Yersinia*-resistant C57BL/6 mice can be rendered *Yersinia*-susceptible by neutralizing IFN-γ *in vivo* with monoclonal antibodies (10). Conversely, *Yersinia*-susceptible BALB/c mice can be rendered resistant by treatment with IFN-γ. In keeping with these results, it was found that administration of neutralizing anti-IL-4 antibodies also rendered BALB/c mice resistant to *Yersinia* infection (10). From these data it can be concluded

that IFN-γ is a central protective cytokine in *Yersinia* infection. In addition experiments with cytokine- or cytokine receptor-deficient mice clearly demonstrated that the cytokines TNF-α, IL-12, IL-18 and IFN-γ are all essential for control of *Yersinia* infection (26,28,29,81).

In contrast to T cell responses, protective antibodies recognize the outer membrane protein YadA of *Y. enterocolitica* (214). In *Y. pestis* infections, antibodies against LcrV, F1 antigen and YopD have been demonstrated to be protective. Together, these data suggest that different antigens are possibly involved in protective cellular and humoral immune responses.

1.5 Objectives and goals

By means of its plasmid-encoded pathogenicity factors including the type three secretion system (TTSS) and YadA *Y. enterocolitica* is able to evade the innate immune system like phagocytosis by PMNs and macrophages and lysis by complement. Therefore the production of cytokines like TNF-α, IL-12, IL-18 and IFN-γ as well as the activation of CD4 T cells and macrophages are essential to control *Yersinia* infection. Herein, we addressed the question whether and how *Y. enterocolitica* also evade adaptive immunity. As DCs build a link between innate and adaptive immune responses and are able to prime naive CD4 T cells these cells could play an important role to overcome *Yersinia* infection. Therefore we investigated whether and how *Y. enterocolitica* affects the immunogenic properties of DCs. To accomplish this, DCs were infected with *Y. enterocolitica* and isogenic mutant strains deficient for plasmid-encoded virulence factors and analysed for cell viability, expression of co-stimulatory molecules, secretion of cytokines and antigen uptake and processing by DCs. Finally, we tested if *Y. enterocolitica* infection alters the ability of DCs to activate CD4 T cells.

2 Material and Methods

2.1 Mice

BALB/c mice were purchased from Harlan Winkelmann (Borchen, Germany). DO11.10 (86), C57BL/6x129Sv and TLR2$^{-/-}$xTLR4$^{-/-}$ mice (110) , provided by C. Kirschning, were maintained under specific pathogen-free conditions in our breeding facility. The experiments were performed using 6 to 10-week-old female mice.

2.2 Bacterial strains and growth conditions

The *Y. enterocolitica* strains used in this study are listed in table 2.1.

Table 2.1: *Yersinia enterocolitica* mutant strains used in this study

Designation	Description	References
pYV^{+}	Serotype O:8, *Y.enterocolitica* WA-314; clinical isolate; contains virulence plasmid pYV; Nal^{r}	(79)
pYV^{-}	Serotype O:8, virulence plasmid cured derivative of *Y.enterocolitica* WA-314; Nal^{r}	(79)
pYV$^{+}\Delta$*yopE*	pYV^{-} harbouring pYV *yopE* Δ17-203; Nal^{r}, Kana^{r}	(97)
pYV$^{+}\Delta$*yopH*	pYV^{-} harbouring pYV *yopH* Δ17-203; Nal^{r}, Kana^{r}	(97)
pYV$^{+}\Delta$*yopM*	pYV^{-} harbouring pYV *yopM* Δ17-203; Nal^{r}, Kana^{r}	(97)
pYV$^{+}\Delta$*yopP*	*yopP*-negative mutant, derivative of *Y.enterocolitica* WA-314, insertional inactivation of *yopP*; Nal^{r}, Cm^{r}	(167)
pYV$^{+}\Delta$*yopT*	*yopT*-negative mutant, derivative of *Y.enterocolitica* WA-314, insertional inactivation of *yopT*; Nal^{r}, Cm^{r}	(167)
pTTSS	pYV^{-} harbouring pTTSS encoding for Type III secretion system and YadA; Nal^{r}, Spec^{r}	(167)
pTTSS*yopE*$^{+}$	pTTSS harbouring pBM-*yopE* Nal^{r}, Cm^{r}, Spec^{r}	Provided by E. Bohn
pTTSS*yopH*$^{+}$	pTTSS harbouring pBME53-*yopH*; Secretion of YopE53YopH fusion protein; Nal^{r}, Cm^{r}, Spec^{r}	Provided by E. Bohn
pTTSS*yopM*$^{+}$	pTTSS harbouring pBME53-*yopM*; Secretion of YopE53YopM fusion protein; Nal^{r}, Cm^{r}, Spec^{r}	Provided by E. Bohn
pTTSS*yopP*$^{+}$	pTTSS harbouring pBME53-*yopP*; Secretion of YopE53YopP fusion protein; Nal^{r}, Cm^{r}, Spec^{r}	Provided by E. Bohn
pTTSS*yopT*$^{+}$	pTTSS harbouring pBME53-*yopT*; Secretion of YopE53YopT fusion protein; Nal^{r}, Cm^{r}, Spec^{r}	Provided by E. Bohn

All strains of *Y. enterocolitica* were grown overnight in Luria-Bertani (LB) broth at 27°C supplemented with the appropriate antibiotics (see Tab. 1). A 1:20 dilution of the overnight *Yersinia* culture was incubated for additional 1.5 h at 37°C. The bacteria were washed once with PBS (Invitrogen, Karlsruhe, Germany) and the optical density at 600 nm was determined.

2.3 Cell cultures and infection

Bone marrow-derived DCs and T cells were grown in RPMI 1640 (Biochrom) medium supplemented with 10% FCS (Sigma, Taufkirchen, Germany), 2 mM glutamine (Invitrogen, Karlsruhe, Germany), 100 U/ml penicillin (Invitrogen, Karlsruhe, Germany), 100 µg/ml streptomycin (Invitrogen, Karlsruhe, Germany), 50 µM β-mercaptoethanol (Sigma, Taufkirchen, Germany), 1% (v/v) non-essential amino acids (Biochrom, Berlin, Germany) and 1 mM sodium pyruvate (Biochrom, Berlin, Germany). DCs were prepared according to the protocol of Lutz *et al.* (115). 2 x 10^6 bone marrow cells, flushed from the femurs and tibias of mice, were seeded in 100 mm dishes with 10 ml of medium containing 200 U/ml GM-CSF (produced by mouse myeloma strain P3X63). After 3 days, 10 ml of fresh medium containing 200 U/ml GM-CSF were added to the cultures. After 6 and 8 days, half of the culture supernatant was collected, centrifuged, and the cell pellet was resuspended in 10 ml fresh medium containing GM-CSF and given back to the original plate. At day 8 the slightly attached cells, 65-75 % are $CD11c^+$, were used for the experiments described herein.

For infection DCs were seeded in 48-, 24-, 12-, or 6-well plates (see particular experiment; non tissue culture treated plates, BD Falcon, Germany) in medium without antibiotics and infected with different strains of *Y. enterocolitica* at a multiplicity of infection (MOI) of 10 or stimulated with 1 µg/ml LPS (from *S. typhimurium*, Sigma, Taufkirchen, Germany). The bacteria were sedimented onto the cells at 400 x *g* for 5 min. After 1 h of infection gentamicin (0.1 mg/ml; Sigma, Taufkirchen) was added to kill extracellular bacteria.

2.4 Purification of DO11.10 CD4$^+$ T cells

CD4$^+$-transgenic T cells were isolated from the spleens of 8-week-old Ova-TCR-transgenic DO11.10 (H-2^d) mice. CD4$^+$ T cells were purified by preparing a single cell suspension, followed by lysis of red blood cells (80 mM NH_4Cl, 5 mM $KHCO_3$, 5 mM Na_2EDTA). Using the CD4$^+$ negative T cell isolation kit and the magnetic-activated cell sorter system (Miltenyi Biotech, Bergisch Gladbach, Germany) MHC class II$^+$, B220$^+$ and CD8$^+$ cells were depleted. The resulting T cell preparations, containing 97-99 % CD4$^+$ T cells, were used without further purification.

2.5 T cell proliferation assay

DCs were infected with the indicated strains of *Y. enterocolitica* (1 x 10^6 DCs in 24-well plate). At 1 h post infection 0.1 mg gentamicin/ml and 0.1 mg OVA/ml (Sigma, Taufkirchen, Germany) were added and the cells were incubated at 37°C for another 2 h. Freshly isolated DO11.10 T cells were labelled with CFSE (5-(6)-carboxyfluorescein diacetate N- succinimidyl ester; Molecular Probes/Invitrogen, Karlsruhe, Germany) as previously described (108,117) with minor modifications. 1-2 x 10^7 T cells/ml were washed twice with ice-cold PBS and incubated with CFSE (5 µM) in PBS for 3 min at 37°C. The cells were then washed twice with ice-cold heat-inactivated FCS, resuspended in cell culture medium and used for the experiments. CFSE-labelled T cells (4 x 10^5) were co-incubated with the Ova-loaded DCs (10^5) in 48-well plates. After 4 days of co-incubation, the proliferation rate was analysed by flow cytometry with excitation at 488 nm and the FL 1 detection channel.

2.6 Assessment of apoptosis

2.6.1 Assessment of the mitochondrial transmembrane potential

DCs (4 x 10^5 in 24-well plate) were infected with different *Yersinia* strains as described above. The mitochondrial transmembrane potential ($\Delta\Psi_m$) was analysed using the $\Delta\Psi_m$-specific stain TMRE (Molecular Probes/Invitrogen, Karlsruhe, Germany). TMRE (40 nM) was added at 3.75 h post infection and cells were incubated for further 15 min at 37°C. Thereafter, the cells were additionally stained with propidium iodide (PI, 40 ng/ml) and analysed by flow cytometry using FL-2 channel for TMRE and FL-3 channel for PI.

2.6.2 Assessment of DNA fragmentation

Nicoletti assay: 24 h after infection cells were transferred into FACS tubes (BD Falcon, Germany) and centrifuged at 400 g for 5 min. The pellet was resuspended in 400 µl of Nicoletti reagent (50 µg/ml PI, 0.1% tri-sodium-citrate-2-hydrate, 0.1% Triton X-100, dissolved in H_2O), incubated on ice for 10 min and analysed by flow cytometry in channel FL-3.

2.7 Flow cytometry analyses of adhesion molecules

To examine the regulation of adhesion and co-stimulatory molecules of *Yersinia*-infected DCs, 5 x 10^6 cells (in 6-well plates) were infected as described above. At 3 h post infection the cells were stained with FITC-conjugated α-I-A/I-E (2G9), α-CD80 (16-10A1), α-CD86 (GL1) or α-CD54 (3E2; all from BD PharMingen, Heidelberg, Germany) and propidium iodide (PI; Calbiochem, La Jolla, CA). Samples were analysed on a FACSCalibur flow cytometer (BD Immunocytometry Systems, Germany) gating on the propidium iodide negative cells. Data were analysed with WINMDI version 2.3 software (J. Trotter, Scripps Institute, La Jolla, CA).

2.8 Determination of cytokine production by ELISA

To study the effect of different *Y. enterocolitica* strains on the cytokine release of mouse DCs, cells (1 x 10^6 in 24-well plates) were infected as described above and the supernatants were collected after 6 h for KC ELISA (Duo Set, R&D Systems, Wiesbaden, Germany) and after 24 h for IL-10, IL-12 (p70) and TNF-α ELISA (OptEIA ELISA Set; BD PharMingen, Germany). Cytokine levels were measured according to the manufacturer's instructions.

2.9 Western blot analyses

2.9.1 Iκ-Bα and Iκ-Bβ

DCs (1 x 10^6 in 24-well plates) were infected as described above and at various time points post infection the cells were washed twice with PBS and resuspended in 200 µl of SDS sample buffer (100 mM Tris-HCl pH 6,8, 200 mM 1,4-Dithio-DL-treitol, 4% SDS, 20% glycerol). Extracts were transferred into reaction tubes (Eppendorf, Hamburg, Germany) and boiled for 5 min. Samples (7 µl) were separated by SDS-

PAGE on a 12.5% polyacrylamide gel and electrophoretically transferred to a nitrocellulose membrane (Schleicher & Schüll, München, Germany). Non-specific binding was blocked with washing buffer (10 mM Tris-HCl, 150 mM NaCl, 0.2 % Tween) containing 5% skim milk for 90 min at room temperature. The membrane was incubated overnight at 4°C with either Iκ-Bα (1:3000; sc-371) or Iκ-Bβ (1:3000; sc-946; Santa Cruz, Heidelberg, Germany) polyclonal antibody. After three 5 min washes the membrane was incubated with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin (1:1000; DAKO, Hamburg, Germany) for 1 h at room temperature. After further washing the bands were visualized with an enhanced chemiluminescence detection system (ECL; Amersham Biosciences Europe GmbH, Freiburg, Germany).

2.9.2 Cathepsin S

Lysates of endocytic organelles were prepared as described in active-site-directed labelling (2.12) and stored in liquid nitrogen. After thawing cell lysates were treated 1 min in an ultrasonic bath and 2 µg of protein were resolved by 12.5% SDS-PAGE, transferred to PVDF membrane (Millipore, Bedford, MA, USA) and blocked with washing buffer containing 10% Rotiblock (Roth, Karlsruhe, Germany) over night. The membrane was probed 1h with a 1:7500 dilution of anti-cathepsin S polyclonal antiserum (kindly provided by E. Weber, University of Halle, Germany) at room temperature, followed by washing steps three times 1 min and three times 10 min. Afterwards the membrane was incubated 1h at room temperature with a 1:10.000 dilution of secondary anti-rabbit IgG antibody coupled with peroxidase (Southern Biotech, Birmingham, AL, USA). An Amersham detection Kit (Amersham Pharmacia, Freiburg, Germany) was used to visualize the proteins.

The western blot experiments for cathepsins were performed in cooperation with M. Reich, Medicine II, University of Tübingen.

2.10 Electrophoretic mobility shift assay (EMSA)

DCs (5 x 10^6 in 6-well plates) were infected as described above. After various intervals nuclear extracts were prepared as previously described (180). Aliquots of the supernatant containing nuclear proteins were stored at –80°C. Protein concentrations were determined using the Bradford assay. Oligonucleotide probes were labelled with [γ^{32}P]ATP (Amersham Biosciences, Freiburg, Germany) using T4-

Polynucleotidkinase (New England Biolabs, Frankfurt, Germany) and purified on a NucTrap probe purification column (Stratagene, Amsterdam, Netherlands). The following oligonucleotides were used (Santa Cruz, Heidelberg, Germany):
NF-κB wild type (NF-κBc; 5'-ATGTGAGGGGACTTTCCCAGGC-3'), NF-κB mutant (NF-κBm; 5'-ATGTGAGGcGACTTTCCCAGGC-3';). Nuclear extracts (3-6 µg) were incubated with 30 000 cpm of the ^{32}P-labeled oligonucleotide probe for 45 min on ice in a buffer containing 5% glycerol, 80 mM NaCl, 1 mM DTT, 1 mM EDTA pH 8.0, 10 mM Tris-HCl pH 7.2 and 1 µg dIDC. Antibodies against p50 (sc-1190X) and p65 (sc-372X; Santa Cruz) were included in the binding reaction for supershift analyses. Samples were resolved on a 5% non-denaturating polyacrylamide gel using 0.5 x TBE (25 mM Tris-HCl, 25 mM boric acid, 0.5 mM EDTA) as running buffer. Gels were transferred to Whatman 3M paper (Schleicher & Schüll, München, Germany) and dried under vacuum. Protein binding was assessed by autoradiography.

2.11 Measurement of Ag uptake (FACS & Fluororeader)

DCs (5 x 10^5 in 48-well plates or 3 x 10^6 in 12-well plates) were incubated with Ova-FITC (1 mg/ml or 300µg/ml), transferrin-FITC (Tf, 1 mg/ml) or Lucifer Yellow (LY, 2 mg/ml; all from Molecular Probes/Invitrogen, Karlsruhe, Germany) directly or after infection with *Y. enterocolitica* for 30 min at 37°C. Cells were washed three times in ice cold PBS/2% FCS. For flow cytometry analyses (FACS Calibur, BD Biosciences, Heidelberg, Germany) DCs were incubated with APC-anti-CD11c antibody (HL3, BD Biosciences). Ova-FITC, Tf-FITC, or LY uptake was quantified as mean fluorescence intensity (MFI) of $CD11c^+$ cells. As the excitation of FITC is dependent on pH (considerable fluctuations between pH 5-7,5), we used lysates of DCs of pH 9 to analyse the total fluorescence of FITC. DCs were treated with lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0,5% NP-40 and 5 mM EDTA, pH 9) for 30 min on ice and centrifuged at 13000 x *g* for 30 min. The amount of protein was determined using the BCA Protein Assay (Perbio, Bonn, Germany) according to the manufacturer's instructions. The fluorescence of 50 µg protein of each sample was analysed by a Fluororeader (FLUOstar OPTIMA, BMG LABTECH; Gain 1750, excitation filter 485 nm, emission filter 520 nm). Non-specific FITC signal was assessed by incubating cells in Ova-, Tf-FITC or LY at 0°C. In some conditions, cells were pre-treated with 10 µM cytochalasin D (Sigma-Aldrich, München, Germany), 5 µg/ml mannan (from

Saccharomyces cerevisiae; Sigma-Aldrich) or MAPK inhibitors SP600125 (20 µM; inhibiting JNK), PD98059 (50µM; inhibiting MEK1/2) and SB202190 (2µM; inhibiting p38; all from Merck Biosciences GmbH, Bad Soden, Germany) for 15, 10 or 30 min at 37°C, respectively. For inhibition of Rho GTPases (Rho A, Rac1 and Cdc42) cells were pre-incubated with TcdB10463 (20 ng/ml; kindly provided by I. Just, Hannover, Germany) for 2h.

The experiments performed with the fluororeader were done together with R. Rösemann, Institute for Medical Microbiology and Hygiene, University of Tübingen.

2.12 Active-site-directed labelling

DCs (5 x 10^6 in 6-well plates) were infected as described above. 1h post infection gentamicin was added and the cells were used either directly or after additional 2h at 37°C for further purification. 10 wells were pooled for 1 sample and washed twice with ice cold PBS and one time with fractionation buffer (0.25 M sucrose, 10 mM Tris-HCl pH 6.8). Cells were homogenised for subsequent fractionation and centrifuged at 3000 rpm for 10 min. The supernatant was then centrifuged at 50.000 rpm for 5 min using an ultracentrifuge. The resulting supernatant was again centrifuged at 100.000 rpm for 12 min to obtain a highly purified cytosolic fraction and a pellet containing lysosomes and endosomes. These intact endocytic organelles were then lysed in NP-40 lysis buffer, pH 5 (50 mM sodium acetate, 5 mM $MgCl_2$, 0.5% NP-40) and analysed for protein concentration using the Bio-Rad Bradford reagent (Bio Rad Laboratories GmbH, München, Germany). 2-5 µg/ml total endocytic protein were subjected to active-site-directed labelling of cysteine proteases by incubation with reaction buffer (50 mM citrate/phosphate pH 5.0, 1 mM EDTA, 50 mM DTT) in the presence of 10 µM biotinylated DCSG-0N for 30 min at room temperature. For selective inhibition of proteases, appropriate inhibitors were added 30 min prior to labelling. Reactions were determined by addition of SDS reducing sample buffer and immediate boiling. Samples were resolved by SDS-PAGE and transferred to PVDF. After blocking with PBS/10% skim milk, the membrane was probed with a 1:1000 dilution of streptavidin-horseradish peroxidase (Amersham Biosciences Europe GmbH, Freiburg, Germany) in PBS/0,2% Tween 20 for 60 min followed by five washes with PBS/0,2% Tween 20. ECL (Amersham Biosciences, Freiburg, Germany) was used for visualisation.

The active-site directed labelling experiments were performed in cooperation with M. Reich, Medicine II, University of Tübingen.

2.13 Statistics

The significance of the differences among different groups was determined by the unpaired two-tailed Student's *t* test. *P* values < 0.05 were considered statistically significant.

3 Results

3.1 T cell activation after infection of DCs with *Y. enterocolitica*

T cell responses are required for elimination of *Y. enterocolitica* from infected tissues (13,16). Therefore, T cell proliferation assays were performed to analyse whether *Yersinia* infection alters the capacity of DCs to activate T cells. For this purpose DCs were infected with *Y. enterocolitica* wild type or mutant strains for one hour, then ovalbumin protein (Ova), used as antigen, was added to the DCs. Two hours later the Ova pulsed DCs were washed and co-cultured with Ova-specific CFSE-labelled $CD4^+$ T cells from DO11.10 mice. T cell proliferation was analysed by flow cytometry.

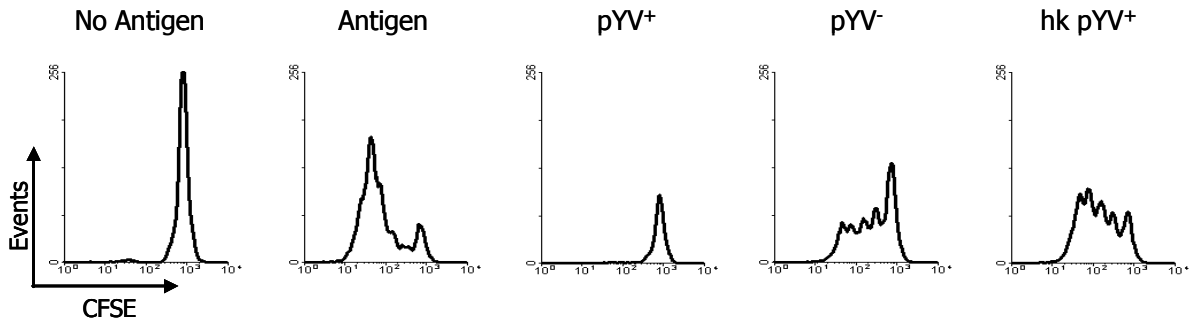

Fig. 3.1: T cell proliferation after co-culture of DCs infected with *Y. enterocolitica*
DCs were incubated with the indicated strains of *Y. enterocolitica* (1h, MOI 10), then pulsed with Ova for 2 h and co-cultured with CFSE labelled $CD4^+$ T cells from Ova TCR transgenic DO11.10 mice. 4 days later T cell proliferation was analysed by flow cytometry. Results are representative of seven experiments.

DCs incubated without Ova and further co-cultured with Ova-specific $CD4^+$ T cells were used as a negative control. This condition did not lead to any T cell proliferation as indicated in Fig. 3.1 by one peak representing $CD4^+$ T cell with the same amount of CFSE. Uninfected DCs pulsed with Ova and incubated with Ova-specific $CD4^+$ T cells were used as a positive control leading to a high proliferation rate demonstrated by series of peaks representing T cells with declining amounts of CFSE. Infection of DCs with wild type *Yersinia* (pYV^+) harbouring the virulence plasmid pYV completely abolished the capacity of DCs to activate T cells (Fig 3.1). In contrast, T cell proliferation was observed when DCs were infected with heat-killed wild type *Yersinia* (hk pYV^+) or with viable plasmid-cured *Y. enterocolitica* (pYV^-), but to a lesser extend

than with the positive control. These results indicate that viable *Y. enterocolitica* may affect DCs in a way which inhibits the ability of DCs to prime CD4 T cells and that factors encoded by the virulence plasmid are essential for this effect.

The virulence plasmid encodes membrane proteins and effector proteins the latter of which are directly translocated via the TTSS into the cytosol of host cells. Our hypothesis was that translocated effector proteins (Yops) might affect intracellular functions of DCs required for their immunostimulatory capacity. To find out which of the *Yersinia* outer proteins (Yops) is responsible for the inhibited T cell proliferation, DCs were infected either with *Yersinia* mutant strains deficient for one single Yop (Fig. 3.2) or mutant strains secreting one single Yop only (Fig. 3.3).

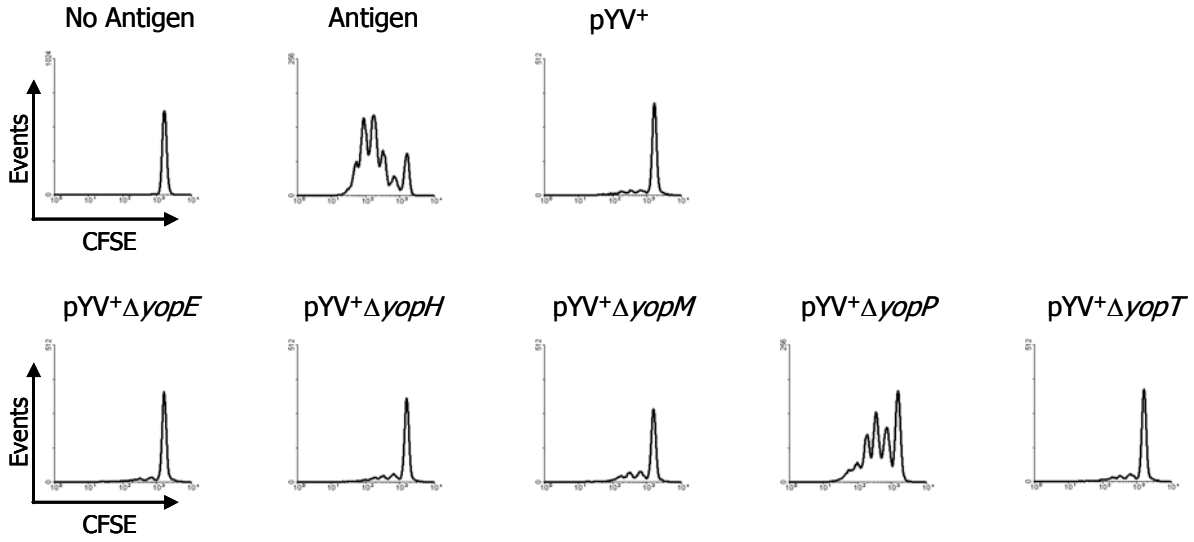

Fig. 3.2: T cell proliferation after co-culture of DCs infected with *Yersinia* mutant strains deficient for single Yops
DCs were incubated with the indicated mutant strains of *Y. enterocolitica*, then pulsed with Ova for 2 h and co-cultured with CFSE labelled $CD4^+$ T cells from Ova TCR transgenic DO11.10 mice. 4 days later T cell proliferation was analysed by flow cytometry.

DCs infected with *Yersinia* mutant strains deficient for YopE, YopH, YopM or YopT completely abolished the capacity of DCs to activate T cells, similar to DCs infected with the wild type strain (pYV^+) (Fig. 3.2). In contrast, DCs infected with the *Yersinia* mutant strain deficient for YopP led to proliferation of Ova-specific $CD4^+$ T cells. This proliferation rate was comparable to that of T cells co-cultured with DCs infected with the plasmid-cured (pYV^-) mutant strain (Fig. 3.1). From these results one can conclude that mainly YopP is responsible for the abolished T cell proliferation

triggered by DCs infected with wild type *Yersinia*. DCs infected with mutants secreting single Yops only, showed similar capability to activate $CD4^+$ T cells as the control mutant pTTSS (Fig. 3.3). Thus, infection of DCs with pTTSSyopP did not lead to decreased T cell proliferation indicating that YopP alone is not able to reduce T cell activation capacity of DCs and that the interplay with other Yops is essential.

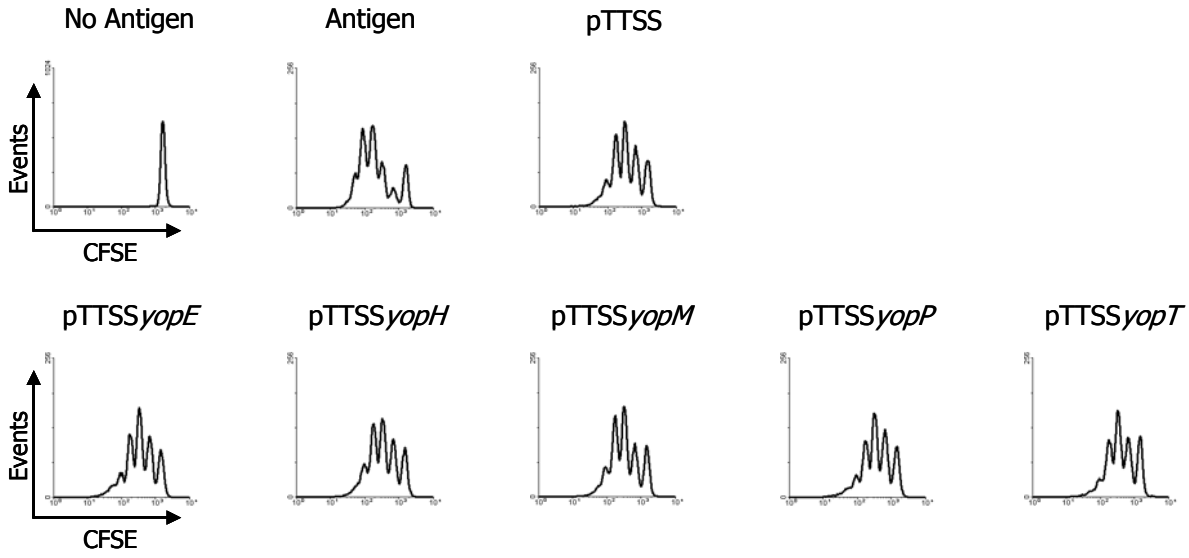

Fig. 3.3: T cell proliferation after co-culture of DCs infected with *Yersinia* mutant strains translocating single Yops
DCs were incubated with the indicated mutant strains of *Y. enterocolitica*, then pulsed with Ova for 2 h and co-cultured with CFSE labelled $CD4^+$ T cells from Ova TCR transgenic DO11.10 mice. 4 days later T cell proliferation was analysed by flow cytometry.

The proliferation rates of infected DCs slightly varied between experiments. This could be due to the inaccurate determination of the amount of bacteria given to the DCs. To confirm this possibility DCs were incubated with different multiplicities of infection (MOI) and T cell proliferation was analysed thereafter. Figure 3.4 demonstrates, that all *Yersinia* mutant strains including plasmid-cured (pYV^-) and wild type heat-killed *Yersinia* were able to inhibit T cell proliferation at high rates of MOI, suggesting that MOIs of 50 cause non-specific toxicity to DCs. In contrast to this, DCs infected with all mutant strains at a MOI of 0,5 or 1 showed no difference in promoting T cell proliferation suggesting that the amount of *Yersinia* effector proteins translocated into DCs by *Y. enterocolitica* is too low to accomplish modulation of DCs. A minimum of 2 to 5 bacteria to DCs is necessary to observe

differences in T cell activation capacity of DCs infected with different *Yersinia* mutant strains.

Together, these data suggest that gene products encoded by the virulence plasmid affect DC functions required to promote T cell proliferation. Several factors like induction of apoptosis, inhibition of maturation, antigen uptake, and processing may affect DCs to prevent T cell proliferation. Therefore, the effect of *Y. enterocolitica* wild type and mutant strains on the function of DCs was analysed in more detail.

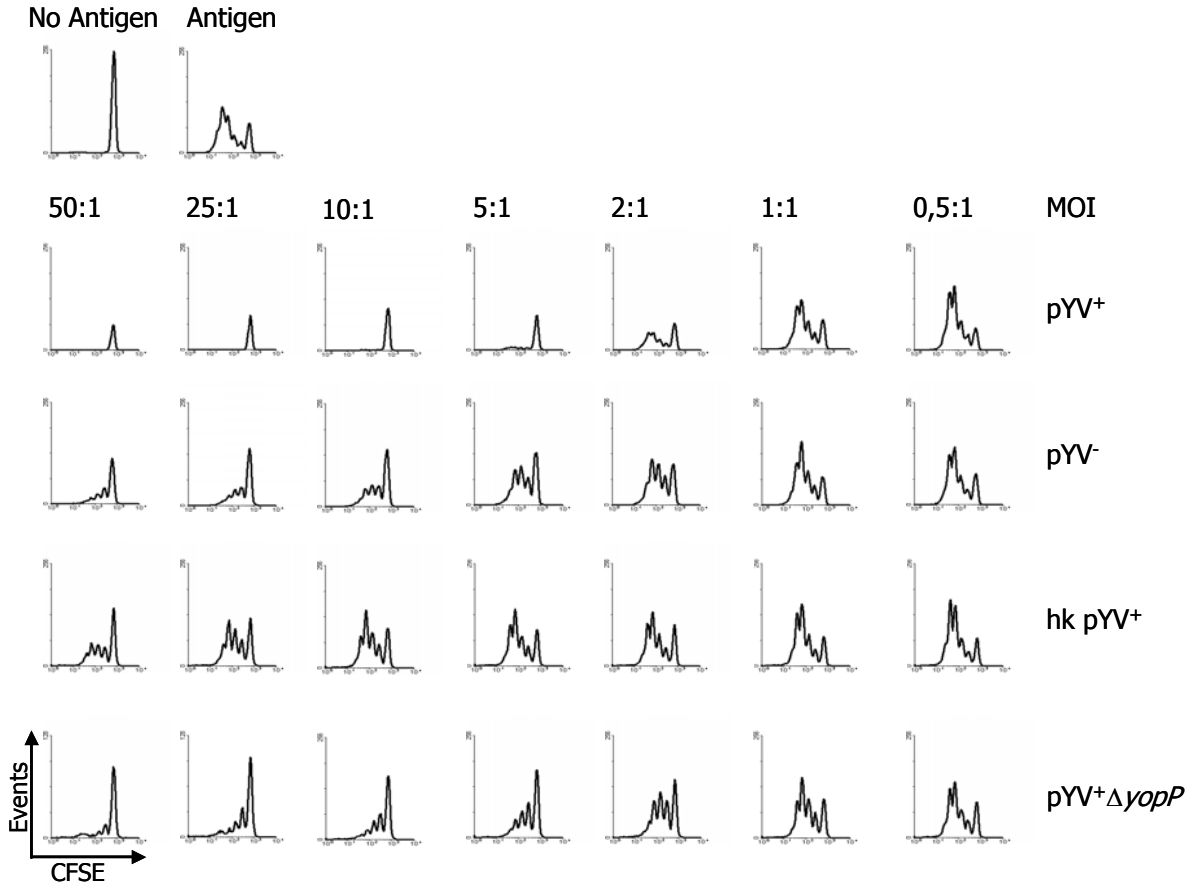

Fig. 3.4: T cell proliferation after co-culture of DCs infected with different multiplicities of infection (MOI) of *Yersinia* mutant strains

DCs were incubated with the indicated mutant strains of *Y. enterocolitica*, then pulsed with Ova for 2 h and co-cultured with CFSE labelled $CD4^+$ T cells from Ova TCR transgenic DO11.10 mice. 4 days later T cell proliferation was analysed by flow cytometry.

3.2 *Y. enterocolitica* induces apoptosis in DCs

Previous studies demonstrated that *Y. enterocolitica* induces apoptosis in mouse macrophages (53,169) but not in human DCs (178). To assess whether *Y. enterocolitica* induces death of mouse DCs, and whether this event might be responsible for the reduced ability of DCs to stimulate T cells, different assays analysing features of apoptosis were performed with DCs infected with *Y. enterocolitica* (Fig. 3.5). The loss of mitochondrial membrane potential ($\Delta\Psi$) is a hallmark of apoptosis. The mitochondrial permeability transition is an important step in the induction of cellular apoptosis. During this process, the electrochemical gradient (referred to as $\Delta\Psi$) across the mitochondrial membrane collapses. The mitochondrial membrane potential ($\Delta\Psi$) was determined by flow cytometry using the mitochondrion selective dye tetramethylrhodamine ethyl ester (TMRE) with an excitation wavelength of 568 nm. In healthy cells TMRE is accumulated by the mitochondria in proportion to the $\Delta\Psi$. In DCs, accumulation of TMRE in the mitochondria results in high fluorescence intensity. In apoptotic cells, where the mitochondrial membrane potential is compromised, TMRE is not accumulated in the mitochondria and therefore the fluorescence intensity of TMRE is low.

To analyse the integrity of cellular membranes, cells were additionally treated with propidium iodide (PI), which is taken up by the cells when the membrane is disrupted. Staurosporine, an inductor of apoptosis, was used a positive control.

Figure 3.5A demonstrates, that four hours after infection with *Y. enterocolitica*, most DCs were TMRE negative (11.6%) and propidium iodide positive (61.7%) while most non infected viable DCs (medium control) were PI negative and TMRE positive (73.2%). These data indicate the loss of mitochondrial membrane potential and damage of the cellular membrane in DCs infected with *Y. enterocolitica* strain (pYV^+).

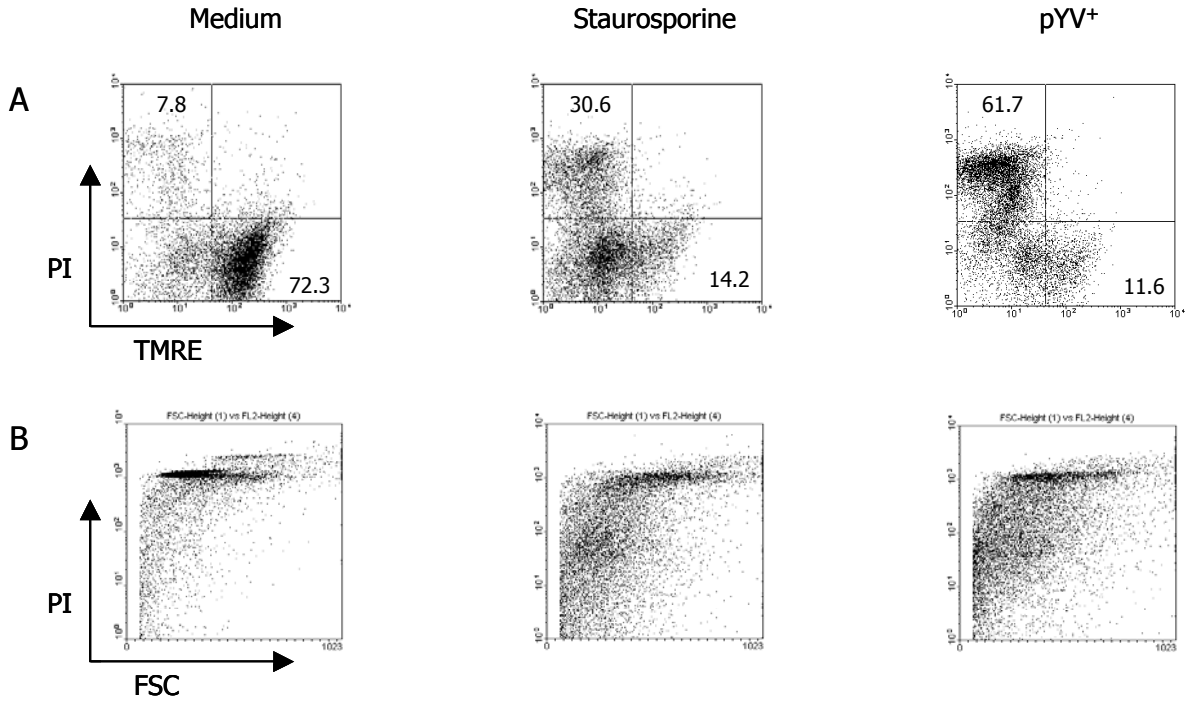

Fig. 3.5: Death of DCs after infection with *Y. enterocolitica*
DCs were left untreated (medium) or incubated with staurosporine or wild type *Y. enterocolitica* (pYV$^+$; MOI 10). 4 h post infection DCs were stained with (A) TMRE and propidium iodide (PI). To assess DNA fragmentation DCs were stained 24 h post infection with PI after cell lysis (B).

To assess DNA content of the nuclei, DCs were lysed and resuspended with Nicoletti reagent including a high concentration of PI. After this treatment PI incorporates in the DNA. The more DNA fragmentation (a hallmark of apoptosis) is obvious in the cells the lesser the PI signal is. DNA fragmentation, a late event in the apoptotic process, was obvious 24 h after infection with the *Y. enterocolitica* wild type strain (pYV$^+$) as indicated by a decreased PI staining in Nicoletti assays (Fig. 3.5B). Therefore, it can be concluded that wild type *Y. enterocolitica* (pYV$^+$) induced death of mouse DCs. To confirm this data electron microscopy analyses were performed with DCs incubated with staurosporine or infected with *Y. enterocolitica* (pYV$^+$). Figure 3.6 shows that DCs treated with staurosporine exhibited typical features of apoptosis like condensation of chromatin and nuclear segmentation. In contrast, predominantly large cells with vacuolized cytoplasm were observed in DCs infected with *Y. enterocolitica*, whereas chromatin condensation was not prominent. These data suggest that *Y. enterocolitica*-mediated cell death of mouse DCs exhibits features of both, apoptosis and necrosis.

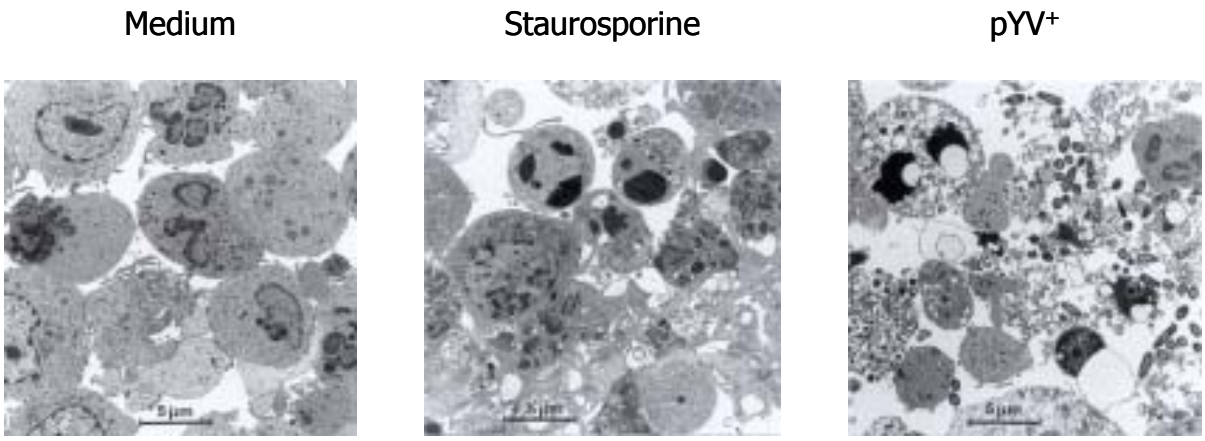

Fig. 3.6: Death of DCs infected with *Y. enterocolitica*
Electron microscopic analyses of DCs incubated with medium demonstrated normal morphology of cells, while treatment with staurosporine or infection with wild type *Y. enterocolitica* (MOI 10, 4 h) induced numerous apoptotic bodies containing cytoplasmic and chromatin remnants.

In contrast to infection with wild type *Y. enterocolitica* (pYV$^+$), apoptosis was not induced by plasmid-cured (pYV$^-$) or by heat-killed wild type *Yersinia* (hk pYV$^+$; Fig. 3.7), indicating a role of effectors encoded by the virulence plasmid. Therefore, DCs infected with mutants deficient for a single Yop as well as mutants secreting single Yops only were analysed for early and late events of apoptosis (Fig. 3.8).

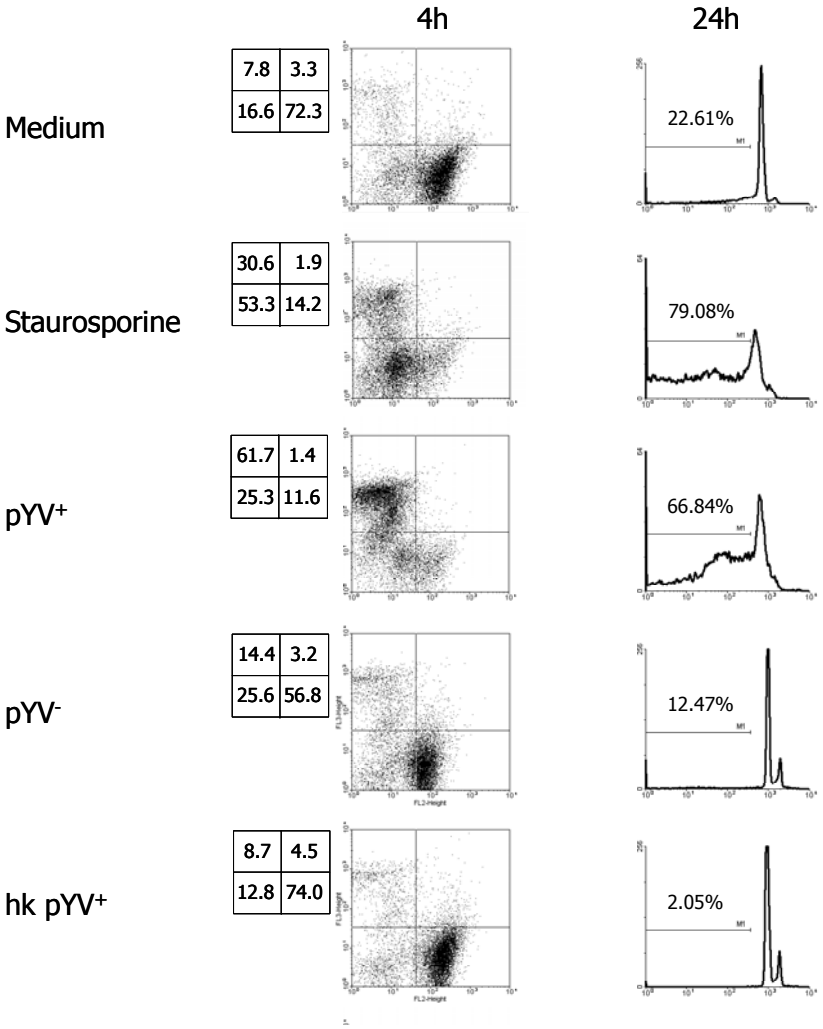

Fig. 3.7: Death of DCs after infection with *Y. enterocolitica*
DCs were left untreated or incubated with staurosporine or different strains of *Y. enterocolitica* (pYV$^+$, pYV$^-$, heat-killed (hk) pYV$^+$, pYV$^+\Delta$*yopP*; MOI 10). 4 h post infection DCs were stained with TMRE and PI (left panel), and 24 h post infection cells were lysed and nuclei were stained with PI only (right panel). Results are representative of three experiments.

The YopP-deficient mutant strain (pYV$^+\Delta$*yopP*) was not able to induce apoptosis in mouse DCs in contrast to all other mutants deficient for a single Yop (Fig. 3.8). According to this only the pTTSS*yopP* mutant strain was able to induce apoptosis in DCs (Fig. 3.8). Therefore, YopP appears to be the most important factor involved in cell death of DCs caused by *Yersinia*.

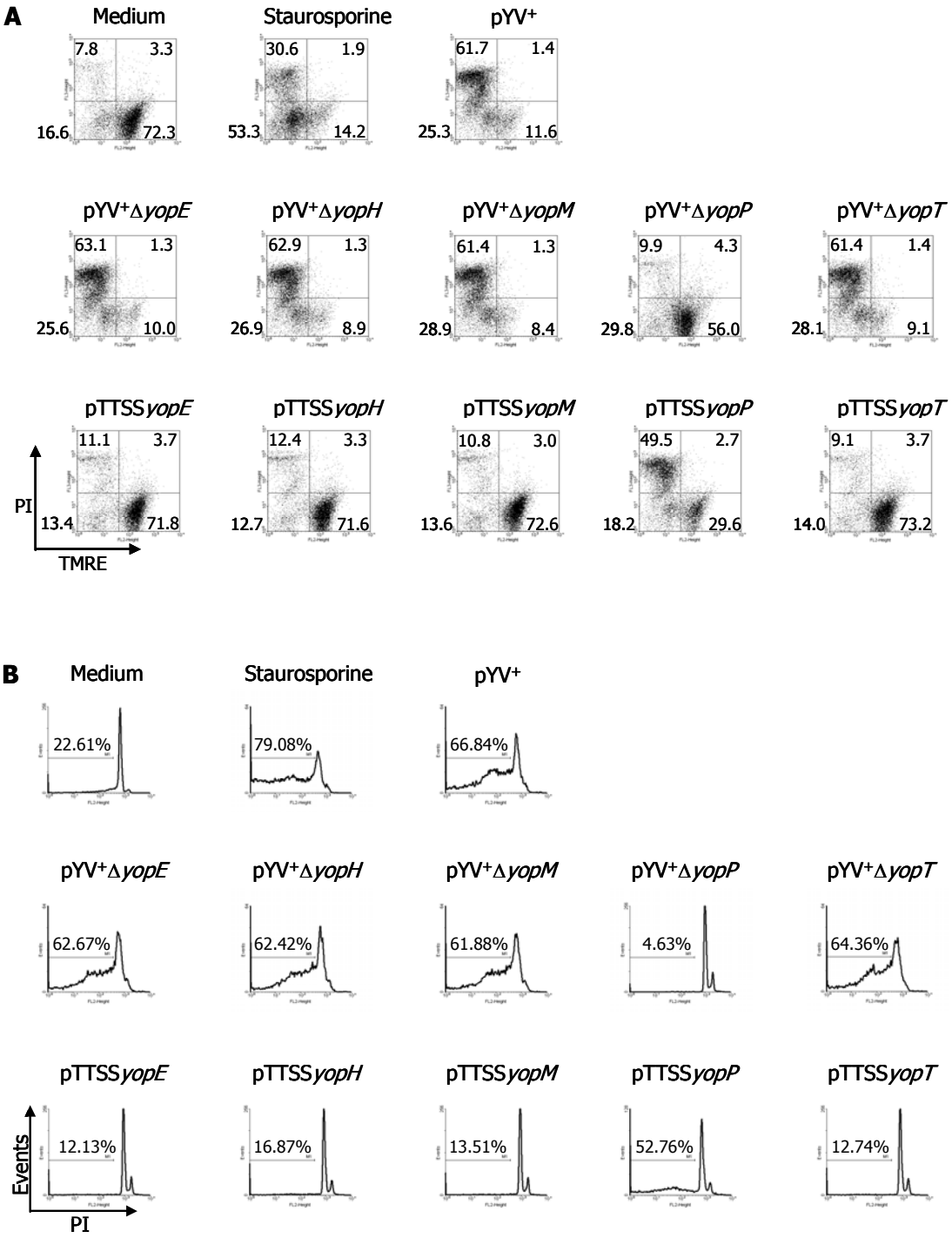

Fig. 3.8: YopP of *Y. enterocolitica* induces death in DCs

DCs were incubated with staurosporine or infected with the wild type or different mutant strains of *Y. enterocolitica* either deficient for a single Yop (A) (pYV$^+$, pYV$^+$Δ*yopE,* pYV$^+$Δ*yopH*, pYV$^+$Δ*yopM*, pYV$^+$Δ*yopP*, pYV$^+$Δ*yopT*) or translocating a single Yop (B) (pTTSS*yopE*, pTTSS*yopH*, pTTSS*yopM*, pTTSS*yopP*, pTTSS*yopT*). (A) 4 h post infection DCs were stained with TMRE and PI and analysed by flow cytometry. Numbers indicate percentages of cells in each quadrant. (B) 24 h post infection DCs were lysed and nuclei were stained with PI (Nicoletti) and analysed by flow cytometry. Numbers indicate percentages of fragmented nuclei. Results are representative of three experiments.

3.3 Surface molecule expression by DCs after infection with *Y. enterocolitica*

Maturation of DCs is accompanied by changes in the expression of surface molecules. To examine whether *Y. enterocolitica* modulates maturation of DCs the expression of molecules, which contribute to immunological synapse formation, including the expression of MHC class II, co-stimulatory, and adhesion molecules (CD80, CD86, ICAM-1), was analysed by flow cytometry.

Three hours after incubation with either LPS, plasmid-cured (pYV^-), or YopP-deficient mutant strain ($pYV^+\Delta yopP$) of *Y. enterocolitica*, expression of MHC class II was markedly increased compared to untreated DCs (Fig. 3.9). Increased MHC class II expression in DCs was also observed after infection with *Y. enterocolitica* wild type (pYV^+) strain, although expression was less compared to DCs infected with *Yersinia* pYV^- and $pYV^+\Delta yopP$. The co-stimulatory molecules CD86 and CD80 as well as the adhesion molecule ICAM-1 (CD54) were strongly expressed on DCs after incubation with LPS or infection with plasmid-cured (pYV^-) or the YopP-deficient mutant strain ($pYV^+\Delta yopP$) of *Y. enterocolitica*, whereas no increased expression was observed after infection with wild type *Y. enterocolitica* (pYV^+; Fig. 3.9). In keeping with these data, infection of DCs with $pYV^+\Delta yopE$, $pYV^+\Delta yopH$, $pYV^+\Delta yopM$ and $pYV^+\Delta yopT$ resulted in reduced upregulation of MHC class II, CD80, CD86 and CD54 (data not shown). Together these results suggest that wild type *Y. enterocolitica* (pYV^+) inhibits maturation of DCs and that YopP accounts for this effect.

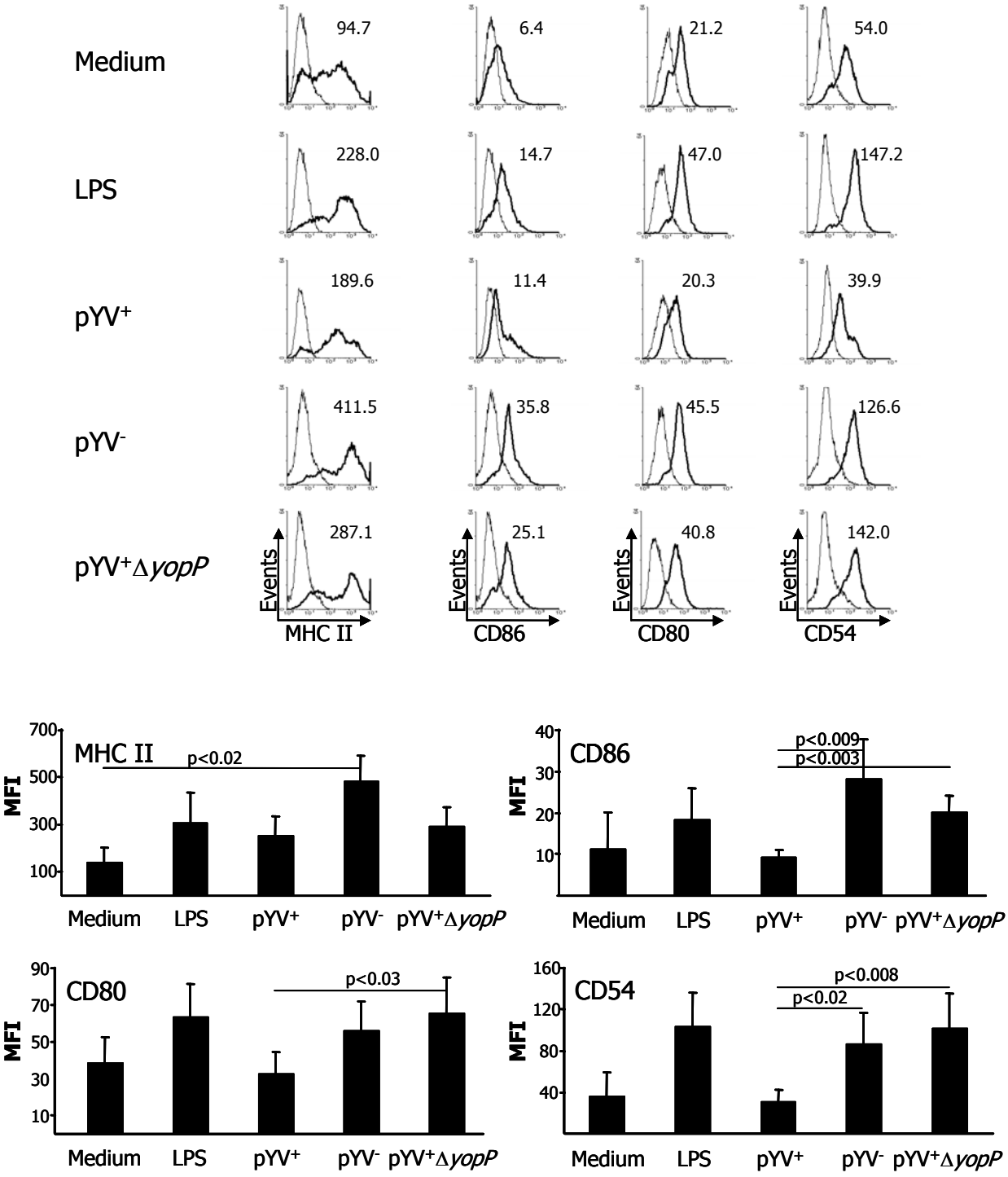

Fig. 3.9: Expression of MHC class II and co-stimulatory molecules by DCs after infection with *Y. enterocolitica*

DCs were incubated with the indicated strains of *Y. enterocolitica*. 3h post infection cells were stained for MHC class II, CD86, CD80 and CD54 (ICAM-1) (thick lines) or isotype-matched control IgG (thin lines) and analysed by flow cytometry gating on the propidium iodide negative population. In the histograms the differences between the mean fluorescence intensities (MFI) values of specific antibodies to isotype controls are indicated. The diagrams show mean fluorescence intensity (MFI) values + SD of 3 independent experiments. Statistically significant differences are indicated.

3.4 Cytokine production after infection of DCs with *Y. enterocolitica*

To analyse whether cytokine release by mouse DCs is altered after infection with *Y. enterocolitica*, DCs were exposed to *Y. enterocolitica* wild type and mutant strains as described above and the cytokines IL-12, IL-10, KC and TNF-α were determined in culture supernatants by ELISA (Fig. 3.10).

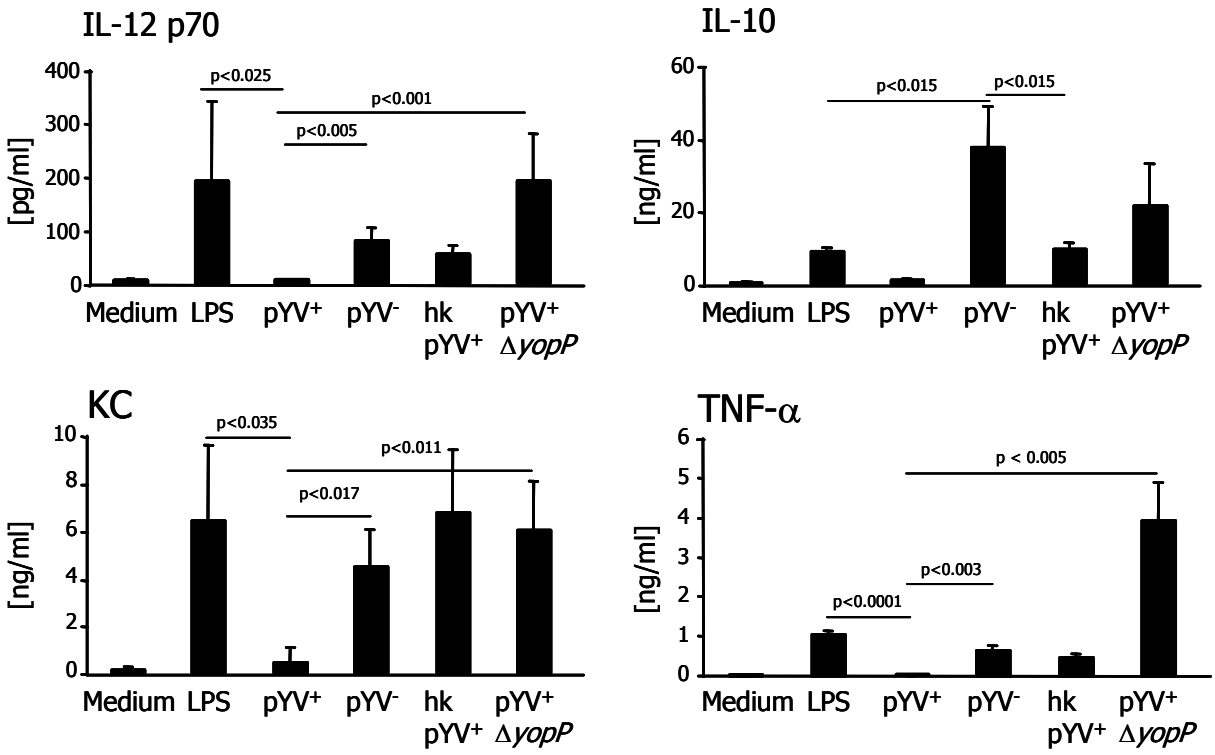

Fig. 3.10: Production of IL-12, IL-10, KC and TNF-α by DCs after infection with *Y. enterocolitica*

DCs were incubated with LPS (1µg/ml) or with the indicated strains of *Y. enterocolitica*. Gentamicin (100 µg/ml) was added 1 h after infection. 3 h (KC) or 23 h (IL-12, IL-10 and TNF-α) later, supernatants were harvested and analysed by ELISA. Data represent the means + SD of three independent experiments. Statistically significant differences are indicated.

Infection of DCs with wild type *Yersinia* (pYV^+) did not result in IL-12 secretion. In contrast, IL-12 secretion was induced by LPS, plasmid-cured (pYV^-) or YopP-deficient ($pYV^+\Delta yopP$) mutant strains of *Y. enterocolitica*. While maximum IL-12 concentrations were observed after treatment of DCs with LPS and YopP-deficient ($pYV^+\Delta yopP$) *Yersinia* mutant strain, the highest concentration of IL-10 was detected after infection with plasmid-cured *Yersinia* (pYV^-). This was significantly higher

compared to incubation with LPS or heat-killed *Yersinia* suggesting a role of invasin or *Yersinia* LPS in stimulating DCs to produce IL-10. No secretion of IL-10 was observed in DCs infected with wild type *Yersinia* (pYV^+), in contrast to DCs infected with YopP-deficient *Yersinia* mutant strain ($pYV^+\Delta yopP$).

IL-8 is known to attract neutrophil granulocytes and also to contribute to T lymphocyte attraction (203). We analysed whether *Y. enterocolitica* may also affect the ability of mouse DCs to secrete KC, the mouse equivalent of IL-8. As shown in Fig. 3.10, incubation of DCs with LPS or *Yersinia* mutant strains (pYV^-, hk pYV^+, $pYV^+\Delta yopP$) resulted in increased secretion of KC. In contrast, secretion of KC was not increased following infection with wild type *Y. enterocolitica* (pYV^+). Similar results were observed regarding the release of TNF-α after *Yersinia* infection. Interestingly, maximum TNF-α was observed in DCs treated with the YopP-deficient mutant strain ($pYV^+\Delta yopP$) (Fig 3.10). In keeping with these data, infection of DCs with $pYV^+\Delta yopE$, $pYV^+\Delta yopH$, $pYV^+\Delta yopM$ or $pYV^+\Delta yopT$ completely inhibited secretion of IL-12, IL-10, KC, and TNF-α (data not shown) comparable with the wild type *Y. enterocolitica* strain (pYV^+). Taken together, these results indicate that *Y. enterocolitica* suppresses cytokine production in DCs predominantly via the action of YopP.

3.5 NF-κB activation after infection of DCs with *Y. enterocolitica*

The transcription factor NF-κB promotes transcription of various immune response genes including those encoding MHC class II, costimulatory molecules (e.g., CD80, DC86) and cytokines (e.g., IL-12, TNF-α) (156). While *Y. enterocolitica* invasin protein was found to activate NF-κB in epithelial cells (181), YopP inhibits the activation of NF-κB, which, in concert with other effects, causes apoptosis of mouse macrophages (166). Therefore, we wanted to know whether *Y. enterocolitica* affects NF-κB activation in DCs. To address this, activation of NF-κB was analysed performing eletrophoretical mobility shift assays (EMSA) of nuclear extracts from DCs infected with wild type or different mutant strains of *Y. enterocolitica* (Fig. 3.11). Nuclear extracts showed low NF-κB-binding activity in untreated DCs. Infection with

plasmid-cured mutant strain (pYV$^-$) or LPS increased NF-κB binding activity in DCs at all indicated time points.

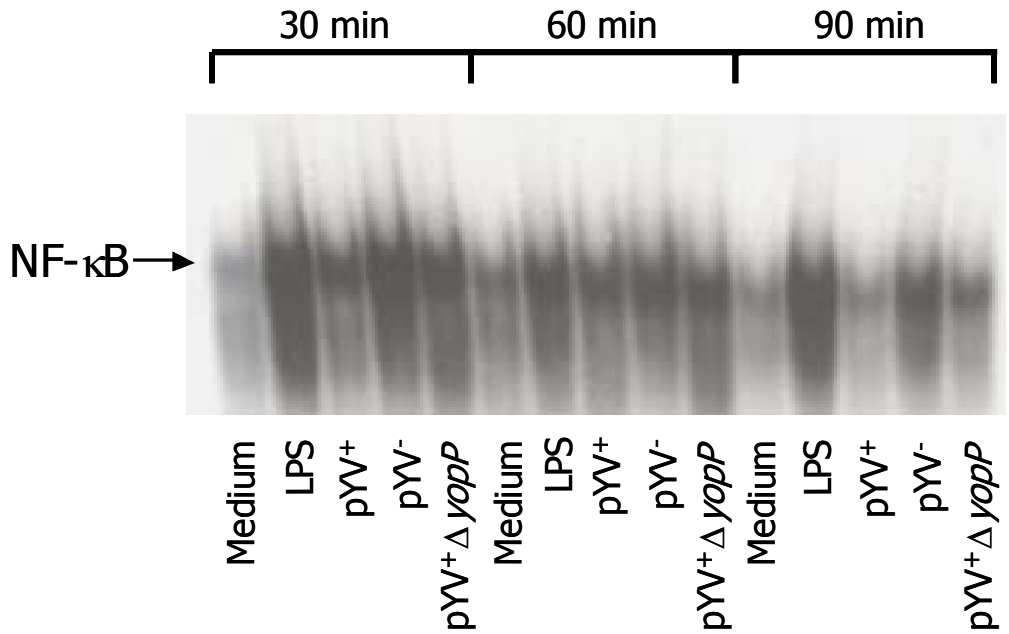

Fig. 3.11: *Y. enterocolitica* induces a transient activation of NF-κB in DCs
Time course of *Yersinia*-induced NF-κB activation. DCs were incubated with LPS (1µg/ml) or with the indicated strains of *Y. enterocolitica*. After 30, 60 and 90 min nuclear extracts were prepared and analysed in gel shift experiments with ^{32}P-labelled probes for NF-κB consensus.

Infection with wild type *Y. enterocolitica* (pYV$^+$) led to an increased NF-κB binding activity 30 and 60 min after infection, but 90 min after infection NF-κB binding activity declined to backround levels. Upon infection with YopP-deficient *Y. enterocolitica* (pYV$^+$Δ*yopP*) NF-κB binding activity was also detectable above backround level 90 min after infection (Fig. 3.11).

Before stimulation, NF-κB is retained in the cytoplasm in an inactive from due to its binding to the inhibitor (IκB) proteins. In response to LPS or other stimuli, IκB is first phosphorylated and then ubiquitinylated and targeted for the proteasome for degradation. This allows NF-κB to translocate to the nucleus and activate transcription of target genes. Therefore, western blot analyses of IκB were performed with DCs infected with different strains of *Y. enterocolitica*. High IκB-α concentrations were observed under control conditions at all indicated time points. Less IκB-α concentrations were observed when DCs were incubated either with LPS

or with *Y. enterocolitica* mutant strain pYV$^-$ 15 to 60 min after infection, indicating degradation of IκB-α (Fig. 3.12). Upon infection with YopP-deficient *Y. enterocolitica* (pYV$^+$Δ*yopP*) IκB-α concentrations declined from 30 to 45 min, but increased 60 min after infection. In contrast, upon infection with wild type *Y. enterocolitica* (pYV$^+$) IκB-α degradation was hardly observed. Since IκB-β concentrations were not changed by infection with *Yersinia*, the alteration of nuclear NF-κB activity appeared to be caused predominantly by changes in IκB-α. Taken together, these results indicate an inhibited activation of NF-κB in DCs infected with *Y. enterocolitica* (pYV$^+$), which is mediated by YopP.

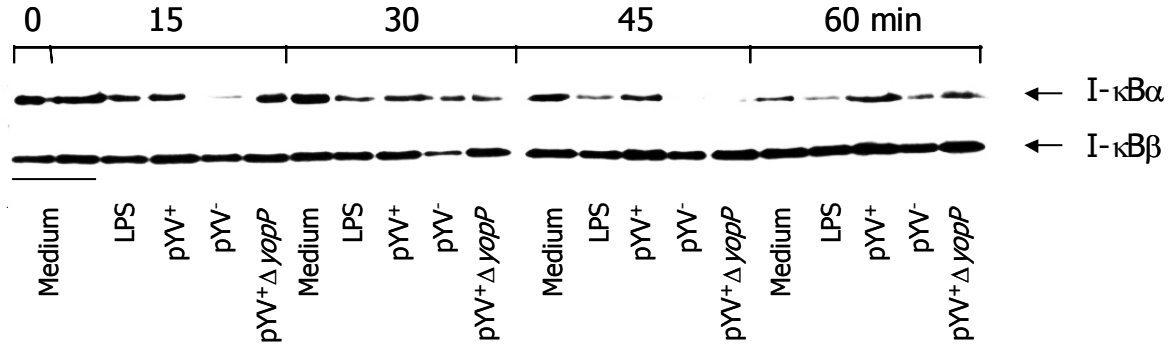

Fig. 3.12: *Y. enterocolitica*-induced degradation of I-κB in DCs
DCs were incubated with LPS (1µg/ml) or with the indicated strains of *Y. enterocolitica*. After 15, 30, 45 and 60 min cells were lysed and analysed in western blot experiments probed for I-κBα (upper panel) and I-κBβ (lower panel). Results are representative of three experiments.

3.6 *Y. enterocolitica* inhibits antigen uptake and processing in DCs

To analyse whether the capacity of DCs to take up and process antigens is impaired by *Y. enterocolitica*, proliferation assays were performed using Ova peptide as well as Ova protein as antigens. Ova peptide can be loaded directly onto MHC class II molecules on the cell surface. Therefore, antigen uptake and processing of Ova peptide by DCs is not required for its presentation by MHC class II molecules to T cells, whereas for the presentation of Ova protein by MHC class II molecules antigen uptake and processing is necessary.

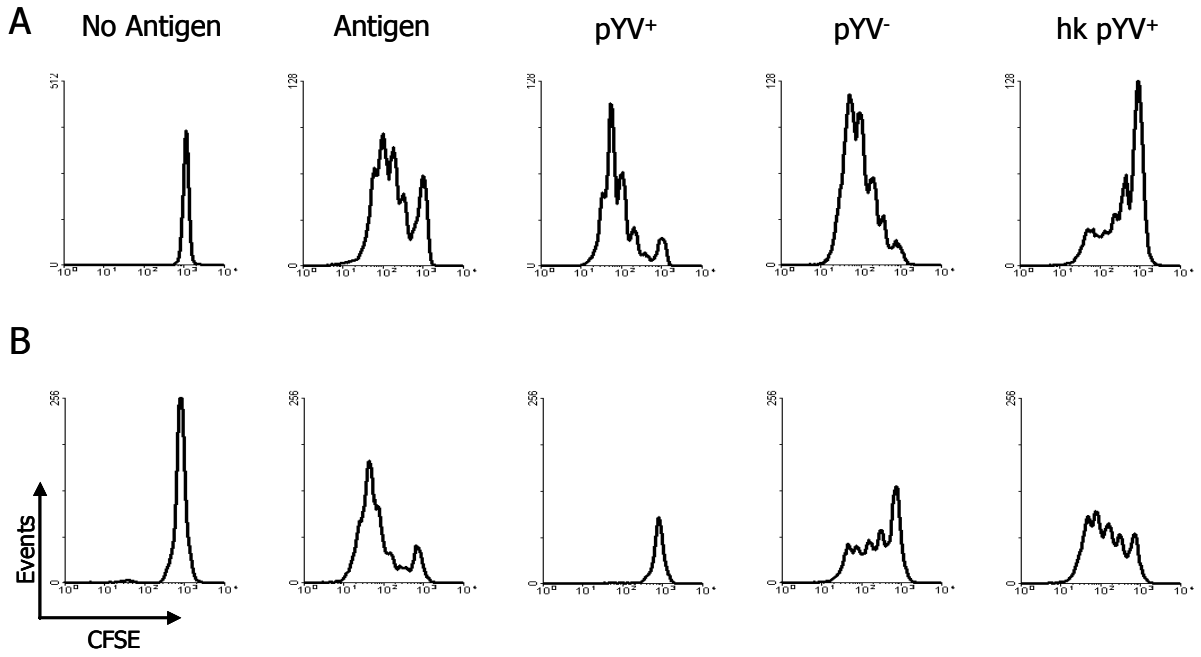

Fig. 3.13: T cell proliferation after co-culture of DCs infected with different *Yersinia* mutant strains and pulsed with either Ova peptide or Ova protein
DCs were incubated with the indicated mutant strains of *Y. enterocolitica*, then pulsed with Ova peptide (A) or Ova protein (B) for 2h and co-cultured with CFSE labelled $CD4^+$ T cells from Ova TCR transgenic DO11.10 mice. 4 days later T cell proliferation was analysed by flow cytometry.

Uninfected DCs pulsed with either Ova peptide (Fig. 3.13A) or Ova protein (Fig. 3.13B) and incubated with Ova-specific $CD4^+$ T cells both led to a high proliferation of the T cells. Infection of DCs with wild type *Yersinia* (pYV^+) completely abolished the capacity of DCs pulsed with Ova protein to activate T cells (Fig 3.13B). In contrast, a high T cell proliferation was observed when DCs were infected with wild type *Yersinia* (pYV^+) and pulsed with Ova peptide. The proliferation rate of Ova-

specific $CD4^+$ T cells co-cultured with DCs either infected with the plasmid-cured *Y. enterocolitica* (pYV^-) mutant strain or the heat-killed *Y. enterocolitica* (hk pYV^+) wild type strain and pulsed with Ova peptide was more or less comparable to DCs pulsed with Ova protein (Fig. 3.13). These results indicate, that *Yersinia* affects antigen uptake and processing of DCs.

3.6.1 *Y. enterocolitica* inhibits uptake of OVA-FITC by DCs

In order to investigate whether *Y. enterocolitica* inhibits antigen uptake DCs were incubated with Ova-FITC and uptake of Ova-FITC was tracked both by FACS and fluororeader. It is well established that after uptake into early endosomes the antigens are transported to the late endosomes and lysosomes (126). During the transport the pH within the vacuoles decreases from 7 to 5. The excitation of the fluorescent dye FITC is highly dependent on pH between 4 to 8. Consequently, Ova-FITC located in early endosomes results in bright fluorescence by FACS analyses while Ova-FITC located in lysosomes should reveal lower fluorescence signals. To overcome this problem lysates of DCs incubated with Ova-FITC were adjusted to pH 9 by means of lysis buffer and analysed by fluororeader, which should result in bright fluorescence. Consequently, Ova-FITC located in both endosomes and lysosomes should be detectable by fluororeader.

A time course of Ova uptake by DCs at 37°C analysed by FACS revealed an increase in Ova-FITC uptake up to 30 min reaching a plateau at 40 min (data not shown). Therefore the following experiments were performed by incubating the DCs for 30 min with Ova-FITC. Ova-FITC was rapidly internalised by DCs incubated at 37°C but not at 0°C resulting in a bright fluorescence after 30 min observed by flow cytometry and fluoreader (Fig. 3.14). Unspecific binding of OVA-FITC to the cell surface was assessed by incubating the DCs with Ova-FITC for 30 min on ice.

DCs were infected with *Y. enterocolitica* wild type (pYV^+) and mutant strains (pYV^-, $pYV^+\Delta yopP$) 1 h prior to incubation with Ova-FITC and subjected to FACS and fluorometric analyses. Fig. 3.14A demonstrates that *Y. enterocolitica* pYV^+, but not the plasmid-cured pYV^- mutant strain, inhibits Ova-FITC uptake by 45% ($p<0,05$) suggesting that pYV encoded virulence factors inhibit antigen uptake in DCs. In DCs infected with YopP-deficient mutant strain ($pYV^+\Delta yopP$) Ova-FITC uptake was not

inhibited (Fig. 3.14A), indicating that YopP is responsible for decreased antigen uptake in DCs infected with wild type *Y. enterocolitica*.

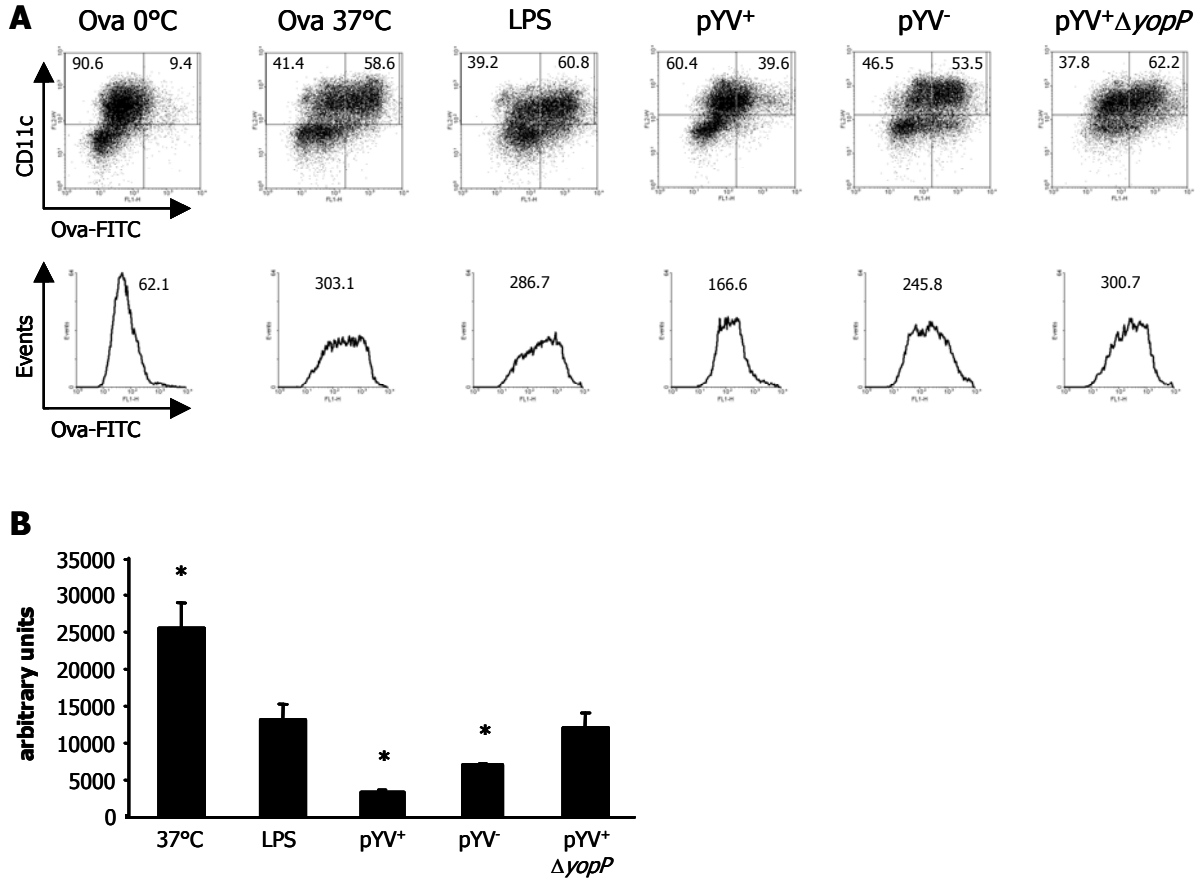

Fig. 3.14: Inhibition of antigen uptake by DCs by *Y. enterocolitica*
DCs were infected with the indicated strains of *Y. enterocolitica* or incubated with LPS for 1 h. Afterwards DCs were incubated in medium containing Ova-FITC and gentamicin for 30 min at 37°C and washed. Unspecific binding was assessed by incubating the DCs with Ova-FITC for 30 min on ice. (A) DCs were stained with an antibody against CD11c, and analysed by flow cytometry. Numbers shown in dot blots indicate percentages of cells in the upper two quadrants. The histograms show Ova-FITC uptake by $CD11c^+$ cells with mean fluorescence intensity (MFI) values. (B) DCs were lysed and analysed by fluorometer. Ova-FITC uptake by fluorometer is shown by arbitrary units. Data represent mean values + SD of 3 individual experiments and * indicate significant differences ($P <$ 0,05) compared to the positive control (37°C).

In comparison to the results obtained by flow cytometry uptake of Ova-FITC by DCs incubated with LPS or infected with the YopP-deficient mutant strain of *Y. enterocolitica* ($pYV^+\Delta yopP$) was reduced by 50% when analysed by fluororeader (Fig. 3.14B). Infection of DCs with the wild type strain and the plasmid-cured mutant strain of *Y. enterocolitica* revealed an inhibition of Ova-FITC uptake by 90% and 70%, respectively (Fig. 3.14B). In summary, similar results were obtained by flow

cytometry and fluororeader except for the fact that the differences in Ova-FITC uptake by DCs incubated with LPS or infected with different mutant strains of *Y. enterocolitica* compared to the positive control were higher when analysed by fluororeader than by flow cytometry. These data confirm the hypothesis that by flow cytometry the presence of Ova-FITC in lysosomes of DCs cannot by determined. In keeping with these data, infection of DCs with pYV$^+\Delta$*yopE*, pYV$^+\Delta$*yopH*, pYV$^+\Delta$*yopM* and pYV$^+\Delta$*yopT* resulted in reduced Ova-FITC uptake (data not shown) comparable with the wild type *Y. enterocolitica* strain (pYV$^+$). As a control, DCs were treated with LPS for 1 h and then incubated with Ova-FITC. The results demonstrate that LPS treatment reduces uptake of Ova-FITC up to 15%, which is in agreement with the concept that activation of DCs results in maturation accompanied by down-regulation of antigen uptake.

3.6.2 MAP kinases are involved in antigen uptake by DCs

In addition to NF-κB signalling, YopP inhibits the MAP kinase (MAPK) signalling pathway by interacting directly with several MAP kinase kinases (MKK) preventing their activation (141,142). However, it was not yet known whether NF-*k*B or MAPKs activation play a role in uptake of antigens in DCs. Previous work provided evidence that selective activation of p38 MAPKs increases endocytic rates in HeLa and BHK cells and suggested that activation of p38 MAPKs allows a more efficient internalisation of cell surface components (36). From this data we hypothesized that there might be a link between the activation of MAPKs and the mechanisms controlling antigen internalisation. To investigate whether MAPKs are involved in antigen uptake, DCs were pre-incubated with the inhibitors SP600125 (JNK), PD98059 (MEK1/2), and SB202190 (p38), or a mixture of these three inhibitors, incubated with Ova-FITC and analysed by FACS and fluororeader (Fig. 3.15 A and B, respectively). Incubation of DCs with inhibitors of JNK, MEK1/2, and p38 reduced antigen uptake by 20-35%. Combination of all three inhibitors reduced antigen uptake by 50-65%. These results suggest that MAPK play an important role in antigen uptake by DCs. The inhibitors were dissolved in DMSO. To exclude effects of DMSO on antigen uptake of Ova-FITC by DCs the cells were treated with similar amounts of DMSO compared to MAPKs inhibitors. The results obtained with DMSO were comparable to the positive control at 37°C.

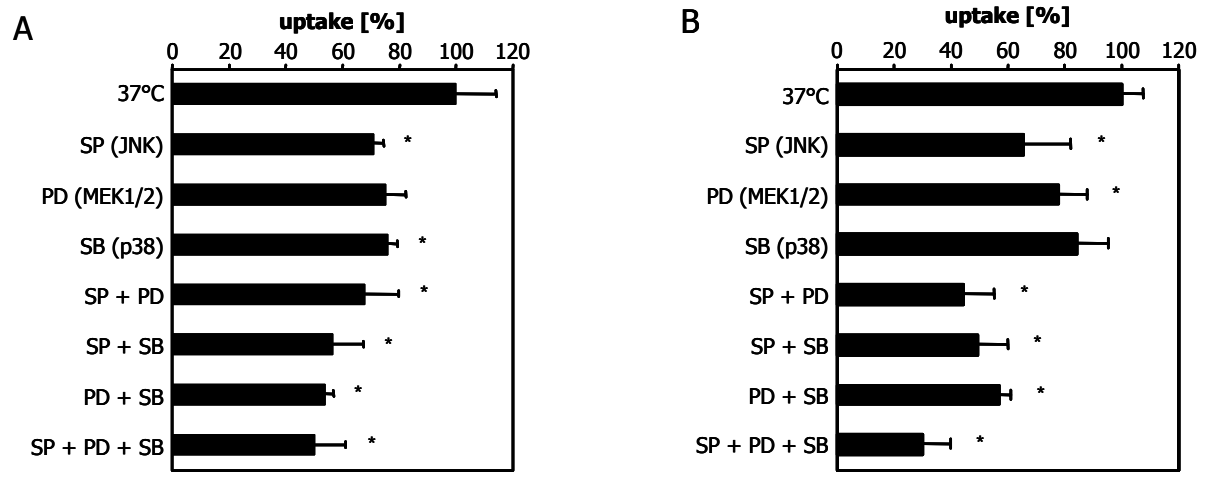

Fig. 3.15: MAPKs are involved in antigen uptake by DCs
DCs were incubated with different MAPK inhibitors [20 µM SP600125 (JNK), 50 µM PD98059 (MEK1/2) and 2 µM SB202190 (p38)] 30 min prior to incubation with Ova-FITC. Then the fluorescence taken up by the cells was analysed by flow cytometry (A) or by fluororeader (B). Ova-FITC uptake is shown as percentages of total uptake of the untreated $CD11c^+$ cells. Data represent mean values + SD of 3 individual experiments and * indicate significant differences ($P < 0,05$) compared to the positive control (37°C).

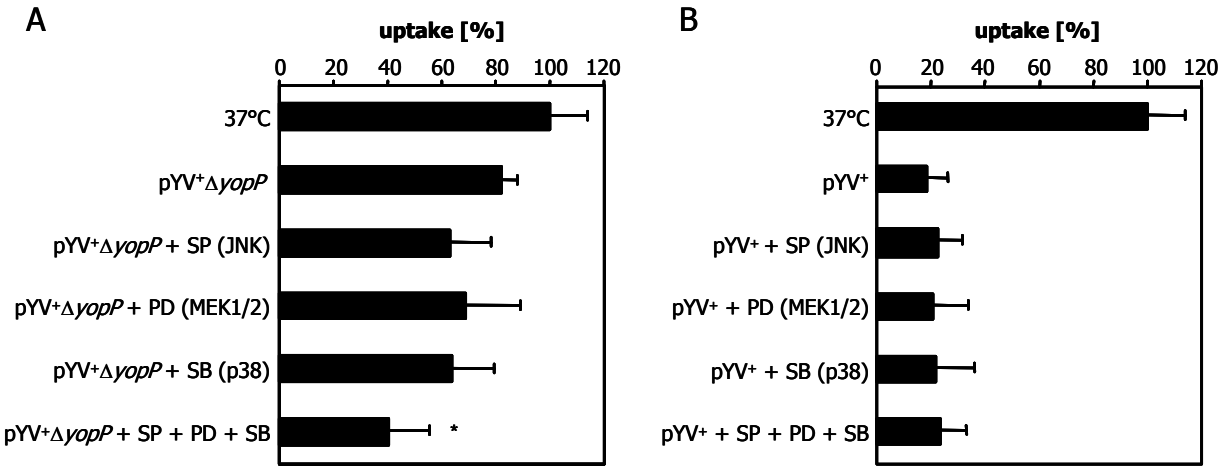

Fig. 3.16: MAPK inhibitors complement inhibition of antigen uptake by DCs infected with YopP-deficient *Yersinia* mutant strain
DCs were infected with the YopP mutant strain $pYV^+\Delta yopP$ (left panel) or the wild type strain pYV^+ (right panel) for 1h. 30 min later MAPK inhibitors [20 µM SP600125 (JNK), 50 µM PD98059 (MEK1/2) and 2 µM SB202190 (p38)] were added to the cells for 30 min. After incubation with Ova-FITC DCs were washed, stained with an antibody against CD11c and the fluorescence taken up by the cells was analysed by flow cytometry. OVA-FITC uptake is shown as percentages of total uptake of the untreated $CD11c^+$ cells. Data represent mean values + SD of 3 individual experiments and * indicate significant differences ($P < 0,05$) compared to DCs infected with $pYV^+\Delta yopP$.

In order to complement the function of YopP, DCs were infected with YopP-deficient mutant strain of *Y. enterocolitica* (pYV$^+\Delta$*yopP*) and simultaneously treated with single MAPK inhibitors or a mixture of the three inhibitors and analysed for Ova-FITC uptake. The results demonstrate that the addition of MAPK inhibitors to DCs infected with *Y. enterocolitica* pYV$^+\Delta$*yopP* leads to a reduction of antigen uptake (Fig. 3.16 A). In contrast, DCs infected with *Y. enterocolitica* pYV$^+$ and treated subsequently with MAPK inhibitors showed no further reduction in Ova-FITC uptake (Fig. 3.16 B).
Therefore, inhibition of Ova-FITC uptake by wild type *Y. enterocolitica* largely depends on YopP and its inhibitory effect on MAPK signalling cascades.

3.6.3 Ova-FITC uptake by DCs is dependent on actin polymerisation but independent of activation of small GTPases

Endocytosis in DCs is controlled by small GTPases. Cdc42 was shown to affect antigen uptake in mouse bone-marrow derived DCs, while Rac1 is involved in endocytosis of mouse splenic DCs (69,220). Uptake of FITC-labelled dextran 40, which was shown to be internalised mainly by clathrin-mediated endocytosis (116), was inhibited by the Rho GTPase inhibitor toxin B of *Clostridium difficile* (190). Ova-FITC is internalised by DCs via both chlathrin-mediated endocytosis and macropinocytosis (116). Macropinocytosis is highly dependent on actin polymerisation (3) and activation of Rho GTPases (56,57) and therefore, can be blocked by cytochalasin D and toxin B from *C. difficile*, respectively. Clathrin-mediated endocytosis, previously referred to as receptor-mediated endocytosis (43), comprises endocytosis of receptors like low-density-lipoprotein receptor (LDLR), transferrin receptor (TfR), macrophage mannose receptor (MMR), Fc receptors, and members of the C-type lectin family (59,152,172,218). Mannan is used to competitively bind to the MMR during Ova-FITC incubation (172). It was shown that actin polymerisation and activation of Rho GTPases is not generally required for clathrin-mediated endocytosis (102,127). To address which of these pathways is involved in the system used in this thesis, inhibitors of both pathways were used and uptake of Ova-FITC was determined (Fig. 3.17).

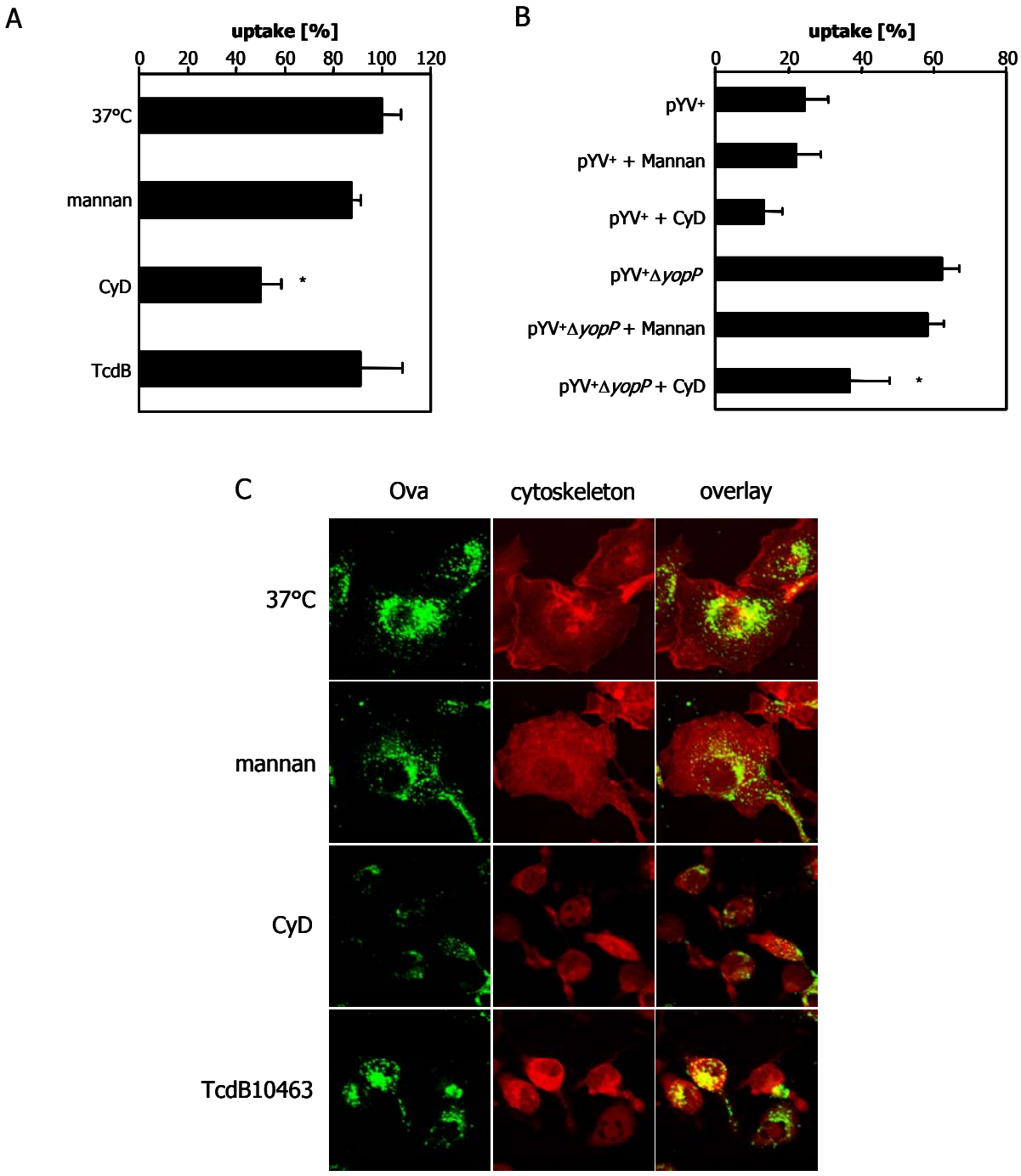

Fig. 3.17: Internalisation of Ova-FITC by DCs depends on actin cytoskeleton but does not require the activity of small GTPases.
DCs were incubated with mannan, cytochalasin D (CyD) or *C. difficile* toxin B 10463 (TcdB) prior to incubation with Ova-FITC and analysed by FACS (A) and confocal microscopy (B). (A) After incubation with Ova-FITC cells were washed, stained with an antibody against CD11c, and the fluorescence taken up by the cells was analysed by flow cytometry. Ova-FITC uptake by untreated $CD11c^+$ cells was set 100%. Data represent mean values + SD of 3 individual experiments and * indicate significant differences ($P < 0,05$) compared to the (A) positive control (37°C) and (B) $pYV^+\Delta yopP$. (C) Confocal microscopy pictures were kindly provided by I. Soldanova. Green: Ova-FITC; red: phalloidin-TRITC.

The most important receptor for the uptake of dextran and mannosylated proteins in human DCs is the macrophage mannose receptor (172). The results pictured in Fig. 3.17 indicated that addition of mannan, which competitively binds to the mannose receptor, does not significantly inhibit Ova-FITC uptake in DCs, indicating that Ova-FITC uptake via the mannose receptor does not play an important role in mouse DCs. Treatment of DCs with cytochalasin D reduced Ova-FITC uptake to 50-60% (Fig. 3.17A). In contrast, incubation of DCs with *C. difficile* toxin B 10463 (TcdB), which inhibits the Rho GTPases RhoA, Rac1 and Cdc42 had no significant effect on Ova-FITC uptake, although with both inhibitors the cytoskeleton of DCs was damaged as demonstrated by confocal microscopy with phalloidin-TRITC staining (Fig. 3.17B). These results indicate that Ova-FITC uptake by DCs is dependent on actin polymerisation but independent of the activation of small GTPases.

Interestingly, CyD additionally reduced Ova-FITC uptake in DCs infected with *Y. enterocolitica* pYV$^{+}\Delta$*yopP* suggesting *Yersinia* inhibits a MAPK dependent pathway for endocytosis and that this pathway is to some degree actin independent (Fig. 3.17 B).

3.6.4 *Y. enterocolitica* and MAPK inhibitors reduce clathrin-mediated endocytosis

To analyse whether clathrin-mediated endocytosis is inhibited by *Y. enterocolitica* transferrin (Tf), which is selectively taken up by the transferrin-receptor (TfR), was used as a marker for clathrin-mediated endocytosis. Clathrin-mediated endocytosis of Tf-TfR complex is constitutively active in DCs. The complex is taken up into early endosomes, where Tf is released from the receptor, which is rapidly recycled to the cell surface.

DCs were infected with different strains of *Y. enterocolitica*, incubated with Tf-FITC and subsequently analysed by flow cytometry (Fig. 3.18). The wild type *Y. enterocolitica* strain (pYV^{+}) inhibited uptake of Tf-FITC in DCs by 35%, compared to the YopP-deficient mutant strain (pYV$^{+}\Delta$*yopP*), which increased Tf-FITC uptake in DCs by 45%. Incubation of DCs with different MAPK inhibitors affecting JNK, MEK1/2, or p38 reduced Tf-FITC uptake by 20-40% indicating that MAPK play also an important role in internalisation of the transferrin receptor.

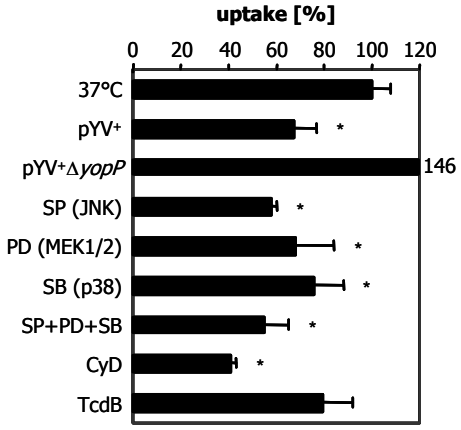

Fig. 3.18: Receptor-mediated endocytosis of transferrin by DCs is reduced by *Y. enterocolitica* and inhibitors of MAPK.
DCs were incubated with different MAPK inhibitors [SP600125 (JNK), PD98059 (MEK1/2) and SB202190 (p38)], cytochalasin D (CyD) or *Clostridium diffifícle* toxin B (TcdB) or infected with *Yersinia* wild type (pYV^+) or with the YopP-deficient mutant strain ($pYV^+\Delta yopP$). After incubation with Tf-FITC DCs were washed, stained with an antibody against CD11c, and the fluorescence taken up by the cells was analysed by flow cytometry. Tf-FITC uptake is shown as percentage of total uptake by untreated $CD11c^+$ cells. Data represent mean values + SD of 3 individual experiments and * indicate significant differences ($P < 0,05$) compared to untreated DCs.

Cytochalasin D reduced Tf-FITC uptake by DCs to 50% (Fig. 3.18), whereas inhibition of Rho GTPases by toxin B had no significant effect on Tf-FITC uptake (Fig. 3.18). From these results one can conclude that *Y. enterocolitica* inhibits antigen uptake via clathrin-mediated endocytosis.

3.6.5 *Y. enterocolitica* inhibits macropinocytosis in a YopP-independent manner

Immature, but not mature, DCs constitutively internalise high amounts of soluble antigen via macropinocytosis. Lucifer yellow (LY) is internalised by DCs via macropinocytosis and can easily be analysed by its property to emit fluorescence. To analyse the influence of *Y. enterocolitica* on macropinocytosis, DCs were infected with different strains of *Y. enterocolitica*, incubated with LY, and analysed by flow cytometry (Fig. 3.19). TcdB inhibits RhoA, Rac1, and Cdc42 as well as CyD, which inhibits actin polymerisation, were used as positive controls to block macropinocytosis. In fact, macropinocytosis in DCs is highly dependent on the action of the RhoGTPases Cdc42, and Rac1 as well as actin polymerisation (69,220). The results revealed that the uptake of LY in DCs incubated with TcdB and CyD was

reduced to 90 and 60%, respectively, suggesting that actin polymerisation and activation of Rho GTPases is crucial for uptake of LY in DCs. The wild type *Y. enterocolitica* strain (pYV$^+$) inhibited uptake of LY by 70% (Fig. 3.19). However, similar results were also obtained with the YopP-deficient mutant strain (pYV$^+\Delta$*yopP*), indicating that inhibition of macropinocytosis by *Y. enterocolitica* is YopP independent. In agreement with this result, incubation of DCs with a mixture of inhibitors of MAPKs JNK, MEK1/2, and p38 did not significantly reduce uptake of LY suggesting that MAPKs do not play an important role in macropinocytosis of mouse DCs.

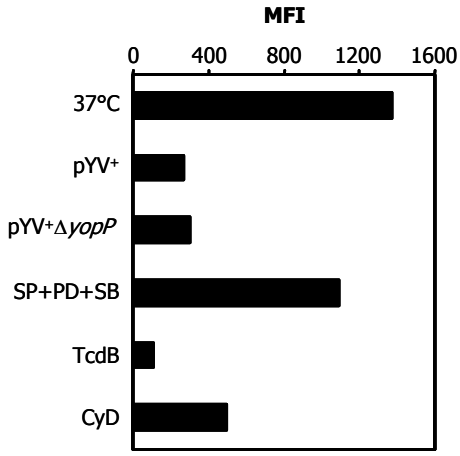

Fig. 3.19: Macropinocytosis of lucifer yellow (LY) in DCs is reduced by *Y. enterocolitica* but not by inhibitors of MAPK.
DCs were incubated with a mixture of different MAPK inhibitors [SP600125 (JNK), PD98059 (MEK1/2) and SB202190 (p38)], cytochalasin D (CyD) or *Clostridium difficile* toxin B (TcdB) or infected with *Yersinia* wild type (pYV$^+$) or with the YopP-deficient mutant strain (pYV$^+\Delta$*yopP*). After incubation with LY DCs were washed, stained with an antibody against CD11c, and the fluorescence taken up by the cells was analysed by flow cytometry. LY uptake is shown as mean fluorescence intensities (MFI) and the data are representative of 3 individual experiments.

Table 3: Inhibition of uptake of Ova-FITC, transferrin-FITC and lucifer yellow by various inhibitors or *Y. enterocolitica*

	Mannan	CyD	TcdB	MAPKs	pYV$^+$	YopP
Ova-FITC	-	+	-	+	+	+
Tf-FITC ≡ clathrin-mediated endocytosis	-	+	-	+	+	+
LY ≡ macropinocytosis	n.d.	+	+	-	+	-

From the aforementioned results it can be concluded that: (i) uptake of Ova-FITC by mouse DCs is mediated by both clathrin-mediated endocytosis (major mechanism) and macropinocytosis (minor mechanism); (ii) MAPKs are involved in clathrin-mediated endocytosis; (iii) *Y. enterocolitica* inhibits both clathrin-mediated endocytosis and macropinocytosis; and (iv) YopP of *Y. enterocolitica* inhibits clathrin-mediated endocytosis.

3.6.6 Inhibition of Ova uptake by *Y. enterocolitica* and MAPK inhibitors is independent of TLR2 and TLR4

Recently it was shown by West *et al.* (221) that TLR ligands acutely stimulate macropinocytosis of Dextran-FITC. This effect was observed only transiently at 30 to 45 min upon TLR activation and a pulse of 10 min with Dextran-FITC and requires p38 and ERK activation. To address whether the reduced uptake of Ova by DCs incubated with *Y. enterocolitica* or MAPK inhibitors is due to the inhibition of MAPKs activated via TLR2 and TLR4, we analysed Ova-FITC uptake by DCs from $TLR2^{-/-}$ x $TLR4^{-/-}$ mice. Wild type *Y. enterocolitica* (pYV^{+}) inhibited Ova-FITC uptake by DCs from both wild type (C57BL/6x129Sv) and $TLR2^{-/-}xTLR4^{-/-}$ mice to 70 and 50%, respectively (Fig. 3.20). No significant reduction of Ova-FITC uptake was observed with DCs from both mouse strains infected with the YopP-deficient mutant strain ($pYV^{+}\Delta yopP$). Moreover, both DCs from wild type mice (C57BL/6x129Sv) and $TLR2^{-/-}$ $xTLR4^{-/-}$ mice incubated with a mixture of MAPK inhibitors of JNK, MEK1/2, and p38 showed a reduced Ova uptake by 65%. These results are similar to those obtained from DCs of BALB/c mice (Fig. 3.14 and 3.15) and demonstrate, that inhibition of Ova uptake by DCs infected with *Y. enterocolitica* or incubated with inhibitors of MAPKs is independent of the TLR2 and TLR4 signalling pathway. Similar results were observed using DCs from $MyD88^{-/-}$ and $TRIF^{-/-}$ mice (data not shown).

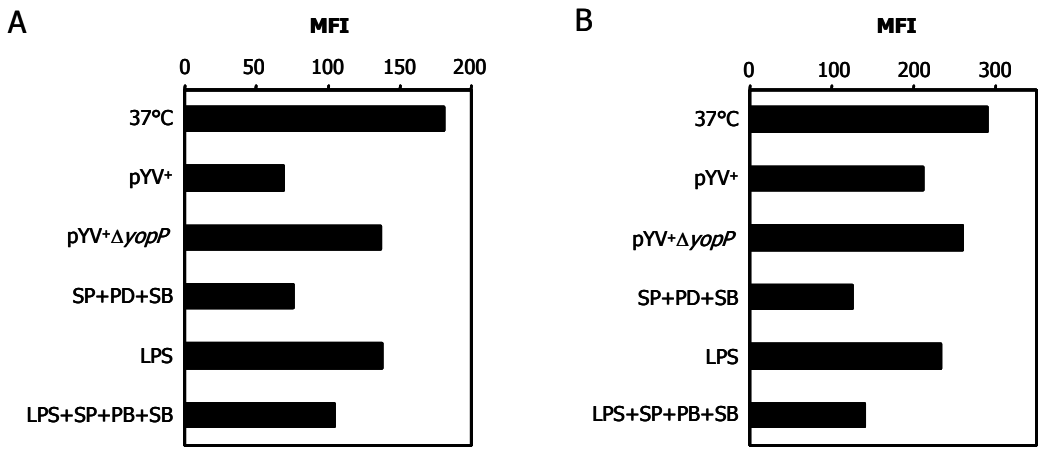

Fig. 3.20: *Y. enterocolitica* and inhibitors of MAPKs reduce uptake of Ova-FITC by DCs from TLR2$^{-/-}$xTLR4$^{-/-}$ mice.
DCs from wild type C57BL/6x129Sv (A) and TLR2$^{-/-}$xTLR4$^{-/-}$ (B) mice were incubated with LPS, a mixture of different MAPK inhibitors [SP600125 (JNK), PD98059 (MEK1/2) and SB202190 (p38)], or infected with *Yersinia* wild type (pYV^{+}) or with the YopP-deficient mutant strain (pYV^{+}Δ*yopP*). After incubation with Ova-FITC DCs were washed, stained with an antibody against CD11c, and the fluorescence taken up by the cells was analysed by flow cytometry. Ova uptake is shown as mean fluorescence intensities (MFI) and the data are representative of 3 individual experiments.

3.6.7 Inhibition of MAPKs in DCs reduces their ability to stimulate T cells

The results obtained in chapter 3.6.2 and 3.6.4 revealed that inhibition of MAPK pathways leads to reduced uptake of Ova as well as Tf. To address whether this reduced antigen uptake has any functional consequences on the capacity of DCs to stimulate T cells proliferation assays were performed. For this purpose DCs were incubated with different MAPK inhibitors prior to incubation with Ova protein. Then, CFSE-labelled T cells were added and T cell proliferation was determined by FACS.

Fig. 3.21 shows that T cell proliferation after co-culture of DCs incubated with MAPK inhibitors was inhibited (19 – 35% proliferated T cells) compared to the positive control (47% proliferated T cells). Inhibition of all three MAPK signalling pathways led to an almost complete inhibition of T cell proliferation compared to untreated DCs.

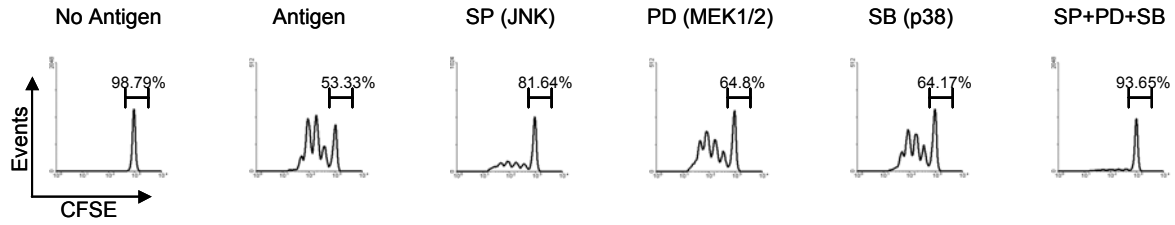

Fig. 3.21: T cell proliferation after co-culture of DCs incubated with MAPK inhibitors
DCs were incubated with the indicated inhibitors of MAPKs (30 min), then pulsed with Ova for 2 h, washed, and co-cultured with CFSE labelled $CD4^+$ T cells from Ova TCR transgenic DO11.10 mice. 4 days later T cell proliferation was analysed by flow cytometry. Numbers indicate the percentage of unproliferated cells. Results are representative of three experiments.

Therefore, one can conclude that inhibition of MAPKs, which leads to a reduced uptake of Ova, also affects the ability of DCs to stimulate T cells. However, it cannot be excluded that inhibition of MAPKs has additional effects on DCs, which may also affect their ability to trigger T cell proliferation.

3.6.8 *Y. enterocolitica* reduces the amount of enzymatically active cysteine proteases in DCs

Cathepsins are known to be essential for antigen degradation as well as degradation of the Ii to process new MHC class II molecules for peptide loading. To address whether pathogenicity factors of *Y. enterocolitica* are able to inhibit the antigen processing machinery of DCs, the amount of enzymatically active cathepsins (Cats) was analysed by active site-directed labelling. For this purpose cell organelles of DCs were purified after infection with *Y. enterocolitica*. The cell organelles were lysed and incubated with a biotinylated probe directly binding to the catalytically active centre of cysteine proteases. Proteins were separated by SDS-PAGE and active cathepsins visualized by streptavidin blotting. Lysates of monocytes (Mo), from which the sequences of the proteins were determined, were used to assign the bands on the blots to the specific cathepsins. As further controls for the active-site-directed labelling lysates were either boiled or incubated with inhibitors of all cysteine proteases (E64), for Cat B (LHVS) or for Cat S (Ca074) prior to incubation with the active-site-directed probe (Fig. 3.22).

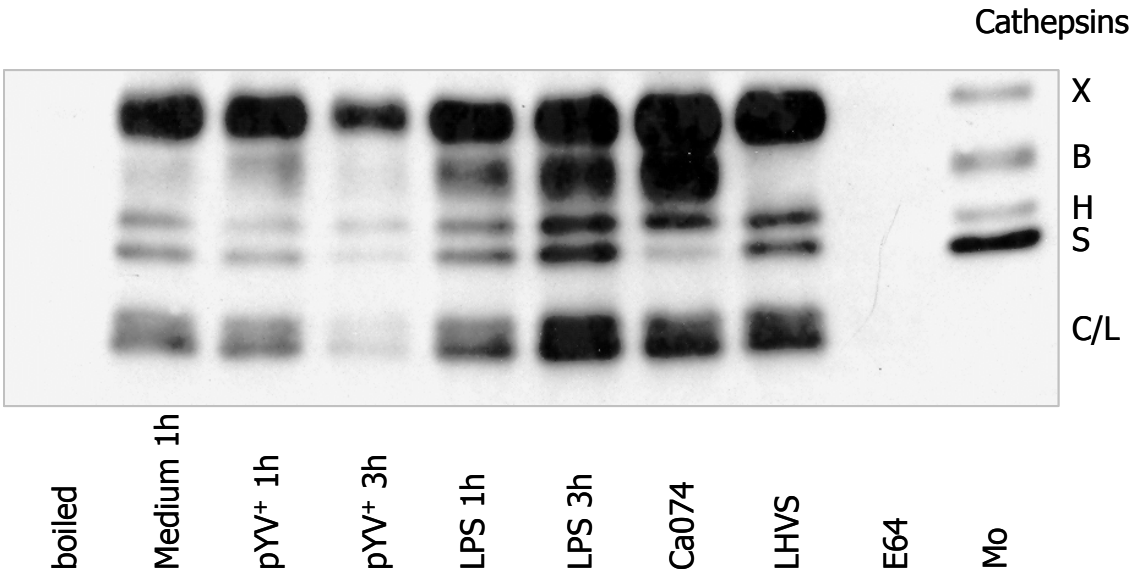

Fig. 3.22: Reduced amount of enzymatically active cathepsins in DCs after infection with *Y. enterocolitica*

DCs were incubated with LPS or infected with the wild type strain of *Y. enterocolitica* for 1 or 3 h. Then the organelles of DCs were prepared, lysed and incubated with a biotinylated active-site directed probe for cysteine proteases. Proteins were separated by SDS-PAGE and active proteases were visualised by streptavidin blotting. The broad-spectrum inhibitor for cysteine proteases E64 as well as the inhibitors of Cat S (Ca074) and Cat B (LHVS) were added 30 min prior to labelling.

The amount of all active cathepsins specific for this probe was reduced 3 h after infection of DCs with the wild type strain of *Y. enterocolitica* (Fig. 3.22) compared to 1 h post infection or untreated DCs. LPS was used as a positive control leading to maturation of DCs and therefore activation of proteases involved in antigen degradation and MHC class II maturation. In agreement with this the amount of active cathepsins highly increased from 1 h to 3 h incubation with LPS compared to untreated DCs. From these data it can be concluded that the amount of active cathepsins is not increased in DCs infected with *Y. enterocolitica*. pYV^+ rather decreased the amount of active cathepsins in DCs although they encounter LPS upon engagement by *Y. enterocolitica*.

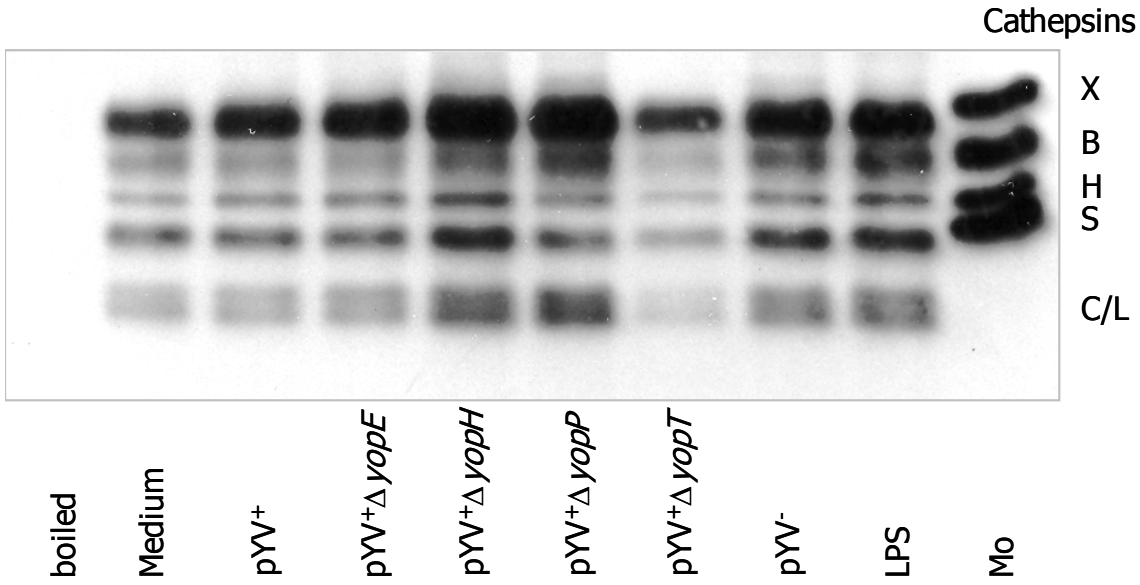

Fig. 3.23: Reduced amount of enzymatically active cathepsins in DCs after infection with different mutant strains of *Y. enterocolitica*
DCs were incubated with LPS or infected with the indicated mutant strains of *Y. enterocolitica* for 3 h. Then the organelles of DCs were prepared, lysed and incubated with a biotinylated active-site directed probe for cysteine proteases. Proteins were separated by SDS-PAGE and active proteases were visualised by streptavidin blotting.

To analyse which pathogenicity factor of *Y. enterocolitica* leads to the reduced amount of active cathepsins, DCs were infected with different mutant strains of *Y. enterocolitica*. Fig. 3.23 shows that infection of DCs with the plasmid-cured mutant strain (pYV^-) led to an increase of active cathepsins in DCs comparable to LPS. Similar results were obtained for the YopH ($pYV^+\Delta$*yopH*) and YopP ($pYV^+\Delta$*yopP*) deficient mutants in this experiment. In 9 of 17 experiments the active site-directed labelling with YopP revealed an increase in the amount of active cathepsins in DCs infected with the YopP-deficient mutant strain, while in 8 of 17 experiments no changes were observed. For DCs infected with the YopH deficient mutant strain an increase in the amount of active cathepsins was observed in 2 of 4 experiments. In contrast, in DCs infected with the YopE and YopT deficient mutant strains the amount of active cathepsins did not increase. These data indicate that plasmid-encoded virulence factors inhibit the increase of active cathepsins in DCs, but it is not clear which Yop is responsible for this effect.

To exclude that this observation is due to non-specific features of the induction of cell death in DCs by YopP of *Y. enterocolitica*, active site-directed labelling experiments were performed by incubating DCs with staurosporine as an inductor of

apoptosis and Z-VAD-fmk as a pan-caspase inhibitor preventing apoptosis. In contrast to DCs infected with *Y. enterocolitica*, DCs incubated with staurosporine highly upregulated the amount of all active cathepsins (Fig. 3.24), indicating that induction of apoptosis in DCs does not lead to a reduced activation of cysteine proteases. After incubation of DCs with Z-VAD-fmk nearly no active cathepsins could be observed, suggesting that Z-VAD-fmk may directly interact with the catalytically active centre of cathepsins.

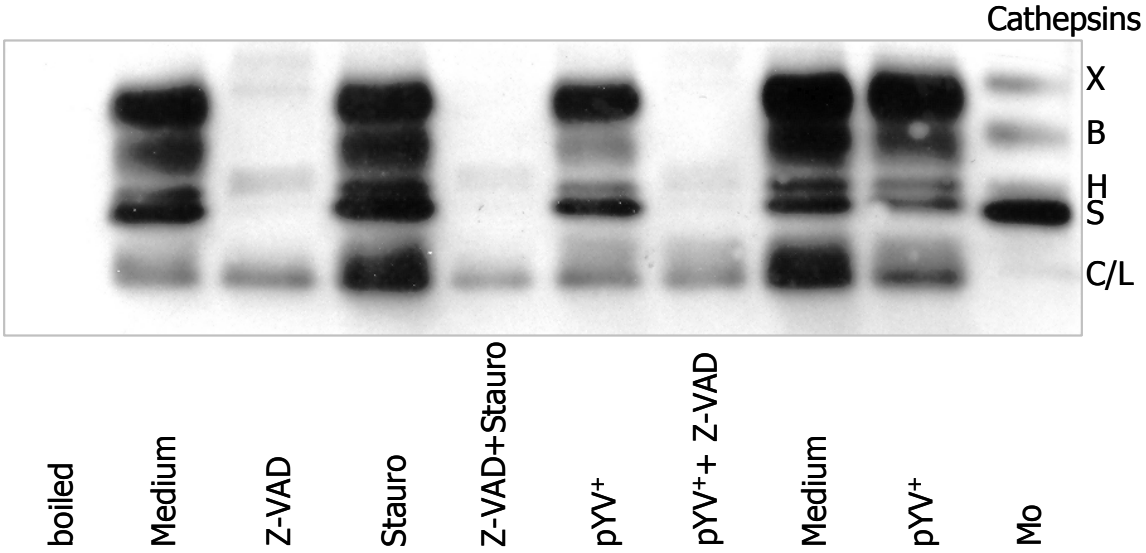

Fig. 3.24: Reduced amount of enzymatically active cathepsins in DCs after infection with *Y. enterocolitica* is independent of cell death
DCs were either incubated with staurosporine, with pan-caspase inhibitor Z-VAD-fmk or infected with the wild type strain of *Y. enterocolitica*. 3 h post infection organelles of DCs were prepared, lysed and incubated with a biotinylated active site-directed probe for cysteine proteases. Proteins were separated by SDS-PAGE and active proteases were visualised by streptavidin blotting.

One possibility for the reduced amount of catalytically active cathepsins could be that *Y. enterocolitica* reduces the total amount of cathepsins. To address this, DCs were infected with *Y. enterocolitica* and different mutant strains and western blot analyses were performed using an antibody for cathepsin S. The data shown in Fig. 3.25 demonstrated that no difference in the amount of total cathepsin S was observed in DCs infected with *Y. enterocolitica* wild type strain (pYV^+), the plasmid-cured mutant strain (pYV^-), or any mutant strain deficient for a single Yop compared to untreated DCs or DCs incubated with LPS (Fig. 3.25).

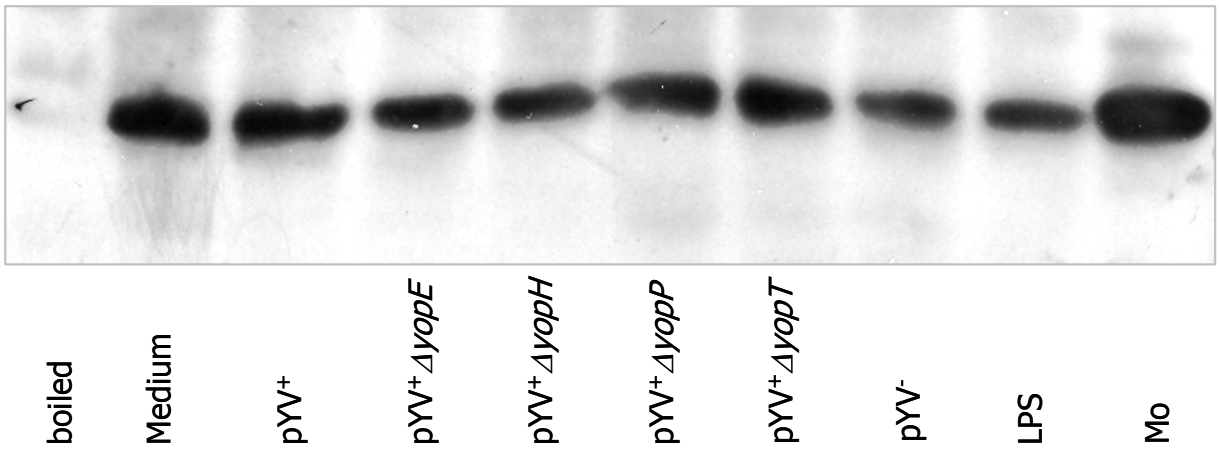

Fig. 3.25: Expression of cathepsin S in DCs after infection with *Y. enterocolitica*
DCs were infected with the indicated strains of *Y. enterocolitica*. 3h post infection organelles of DCs were prepared, lysed and proteins were separated by SDS-PAGE and probed for the expression of cathepsin S by immunoblot.

From these data it can be concluded that plasmid-encoded factors of *Y. enterocolitica* prevent the increase of active cathepsins and that this effect is independent of the induction of apoptosis in DCs by *Y. enterocolitica*.

4 Discussion

CD4 T cells are essential to overcome *Yersinia* infection in mice (13). Therefore, APCs including DCs which present antigen to CD4 T cells via MHC class II molecules might play a crucial role in the defence against infection with *Y. enterocolitica*. Herein, it was elucidated whether and how *Y. enterocolitica* affects DCs in their ability to prime CD4 T cells.

For the stimulation of CD4 T cells several conditions must be fulfilled by the DC: the antigenic peptide must be presented via MHC class II molecules, which requires antigen uptake, processing, and loading onto MHC class II molecules. For the formation of the immunological synapse co-stimulatory as well as adhesion molecules must be expressed on the cell surface in addition to MHC class II-peptide complexes. Furthermore, the production of cytokines like IL-12 and IL-10 is necessary to induce T cell effector differentiation.

The present study demonstrates that *Y. enterocolitica* affects several important functions of mouse DCs including cell viability, maturation, cytokine production, antigen uptake as well as antigen processing. These alterations of DCs by *Y. enterocolitica* obviously contribute to the inhibited antigen-specific CD4 T cell stimulation observed *in vitro*.

4.1 *Y. enterocolitica* inhibits the capacity of DCs to activate CD4 T cells

Proliferation assays performed with DCs infected with *Y. enterocolitica* demonstrated that *Y. enterocolitica* completely blocks the capacity of mouse DCs to activate $CD4^+$ T cells. In contrast, infection of DCs with heat-killed *Yersinia* and the plasmid-cured mutant strain (pYV^-) hardly reduced T cell proliferation compared to the non-infected control DCs. Therefore, chromosomally encoded factors as well as *Yersinia* LPS do not contribute to inhibition of T cell proliferation observed with DCs infected with the *Y. enterocolitica* wild type strain (pYV^+). Therefore, it can be concluded that the main factors leading to a complete inhibition of T cell proliferation observed are encoded by the virulence plasmid *pYV*. T cell proliferation assays performed with DCs infected with different *Yersinia* mutant strains deficient for a single Yop showed that deletion of YopP restored T cell proliferation comparable to that of DCs infected with the

plasmid-cured mutant strain. These data indicate an important role for YopP affecting the capability of DCs to promote T cell proliferation. However, in contrast to this, T cell proliferation was not reduced after infection of DCs using *Yersinia* mutant strains translocating only one Yop e.g. YopP. Analyses of these *Yersinia* mutants for secretion and translocation by western blot revealed that the Yops are translocated and that a defect in translocation of the Yops into the cytosol of the DCs can be excluded (data not shown). Moreover, infection with the *Yersinia* mutant translocating YopP only induced cell death in DCs (Fig. 3.8). Therefore, it is likely that inhibition of T cell proliferation by DCs infected with wild type *Yersinia* is not due to YopP alone, rather it is likely that YopP in concert with other Yops affects DCs' functions.

Recently, similar findings for YopP were observed in an *in vivo* infection model with attenuated *Y. enterocolitica* mutant strains lacking different Yops and translocating a fusion protein of YopE and the listerial antigen LLO by their TTSS. Trülzsch *et al*. could show, that a YopP mutant is able to induce a strong CD8 T cell response to the LLO antigen, whereas mutant strains lacking YopH, YopM, YopE or YopQ did not (206). From this observation they concluded that YopP inhibits the adaptive immune response of the host by suppressing the development of a CD8 T cell response *in vivo* (206).

In contrast to the findings herein, which demonstrate that live *Y. enterocolitica* and factors encoded by the virulence inhibit the ability of DCs to activate CD4 T cells, studies from Schoppet *et al*. revealed that infection of human immature DCs with *Y. enterocolitica* leads only to a transient suppression of T cell stimulation up to five days (178). In a second approach the authors could show that this transient effect is not dependent on viable bacteria (179).

Effects on the ability of DCs to promote T cell proliferation have also been described for *Salmonella enterica* (39). This study reported that intracellular activities of *S. enterica* in DCs suppress antigen dependent T cell proliferation. This effect was dependent on the induction of inducible NO synthase by DCs and on the function of virulence genes in *Salmonella* pathogenicity island 2 (SPI2), a part of the *Salmonella* chromosome encoding e.g. the type three secretion system (39).

4.2 YopP induces cell death of mouse DCs

The most prominent effect of *Y. enterocolitica* on DCs was the induction of cell death. This observation is in contrast to a recent report that provided some evidence that human DCs do not undergo cell death after infection with *Y. enterocolitica* (178). These differences might be due to the different *Yersinia* serotypes which were used in the two studies. In the present study *Y. enterocolitica* serotype O:8 was used. This serotype is known to be a more effective inductor of apoptosis than serotype O:3 (168) which was used in the study from Schoppet *et al*. However, cell death is also observed in a *Yersinia*-infected mouse macrophage cell line (J774), and this effect is caused by YopP (53,169). Consistent with these results we found that in contrast to wild type *Y. enterocolitica* (pYV^+), a YopP-deficient mutant strain ($pYV^+\Delta yopP$) does not induce death of DCs, whereas a mutant strain translocating selectively the effector protein YopP by its TTSS ($pTTSS yopP^+$) was able to induce cell death in mouse DCs. Moreover, it was found that macrophages that had been pre-treated with LPS and thus have been rendered "tolerant" to further LPS stimulation, were much less susceptible to *Yersinia* induced cell death (169). Likewise, we found that mature DCs that had been pre-treated with LPS were less susceptible to *Yersinia* YopP induced cell death than immature DCs (data not shown). This suggests that beside macrophages DCs also undergo cell death upon interaction with pathogenic yersiniae, and YopP appears to be the most important factor for induction of DCs' death.

Exposure of DCs to *Y. enterocolitica* caused a decrease in the mitochondrial membrane potential, DNA fragmentation, and translocation of phosphatidylserine residues from the inner to the outer cellular membrane leaflet, strongly suggesting that *Yersinia* mediated death of DCs is predominantly caused by apoptosis. In the present study the frequency of apoptotic DCs 4 h after infection with *Yersinia* was similar to that observed in experiments with a mouse macrophage cell line (J774) (169). While macrophages were infected with *Yersinia* at a MOI of 50 for 90 min (169), the DCs used in this study were infected at a MOI of 10 for 60 min only. In our experiments higher MOIs caused a rapid and total disruption of DCs suggesting that mouse DCs are more susceptible to *Yersinia*-induced apoptosis than macrophages. In fact, we infected J774 mouse macrophages and DCs with *Y.*

enterocolitica in parallel, and 4 h later survival of macrophages was markedly higher than that of DCs (61% versus 35%; data not shown).

In macrophages, YopP mediated inhibition of NF-κB activation appears to be involved in *Yersinia*-induced apoptosis (166). A biphasic NF-κB activation course was found in *Yersinia*-infected macrophages. NF-κB binding activity was observed after 30 min but was absent 90 min after *Yersinia* infection. In DCs a slight activation of NF-κB at 30 and 60 min was observed even after infection with wild type *Y. enterocolitica* (pYV^+), however, this signal declined markedly after 90 min. The faster prevention of NF-κB signalling in macrophages might result from the higher *Yersinia* MOI used for infection in the experiments carried out by Ruckdeschel *et al*. (166). Despite the fact that nuclear NF-κB activity was obtained for longer periods in *Yersinia* infected DCs than in macrophages, DCs turned out to be more susceptible to killing by *Yersinia* than macrophages. Therefore, at present it is not clear whether and to what extent prevention of NF-κB activation in DCs triggers apoptosis.

Several other pathogens are also able to induce cell death upon interaction with phagocytes. The SPI-1 effector protein SipB of *Salmonella enterica*, translocated via a type III secretion system, was shown to activate caspase-1 in macrophages leading to rapid cell death by a mechanism that has features of both apoptosis and necrosis (133). A similar mechanism for the induction of apoptosis was observed in human macrophages infected with *Shigella flexneri* (83). Infection of human and mouse DCs with *Listeria monocytogenes* revealed similar differences for the two species as observed for *Y. enterocolitica*. In fact, infection of mouse DCs with *L. monocytogenes* induced apoptosis, prevented T cell proliferation, both events were conferred by listeriolysin (50,74) and were not obvious in human DCs infected with *L. monocytogenes* (143).

Additionally, studies from Monack *et al*. revealed a role for YopJ, the analogue of YopP in *Y. pseudotuberculosis*, in the induction of apoptosis in $CD11b^+$ ($Mac\text{-}1^+$) cells *in vivo* (132). After oral inoculation the LD50 of a *Y. pseudotuberculosis* mutant deficient for YopJ increased 64-fold compared to the wild type strain. In fact, 5 days post infection with wild type *Y. pseudotuberculosis* 30% of $Mac\text{-}1^+$ cells in the spleen were TUNEL positive, compared to 2% with the YopJ mutant strain. From these data they conclude that YopJ plays a role in the establishment of a systemic infection by inducing apoptosis.

Preliminary data from *in vivo* experiments in our group showed only low apoptotic $CD11c^+$ cells in the spleen of mice either infected with wild type *Yersinia* or the YopP-deficient mutant strain (data not shown). In addition we observed no significant difference in CFU in the liver after i.v. infection with the wild type *Y. enterocolitica* and the YopP mutant strain. Trültzsch *et al*. found that in mice the YopP mutant strain was slightly attenuated compared to the parental strain (2 fold lower CFU counts in the spleen of mice infected with the YopP mutant strain compared to the wild type pYV^+) (207). This suggests a limited role of YopP for the pathogenicity of *Y. enterocolitica* in the experimental mouse infection model.

Therefore, further experiments are necessary to address whether YopP of *Y. enterocolitica* plays a role in the induction of apoptosis and inhibition of priming CD4 T cell responses in DCs *in vivo*. Furthermore, the mechanism by which YopP induces cell death in mouse DCs has to be elucidated.

4.3 YopP inhibits maturation and cytokine production of DCs

Another assumption of proper T cell activation capacity of DCs is the maturation, including upregulation of MHC class II molecules, costimulatory molecules like CD80, CD86, CD40 as well as adhesion molecules e.g. CD54 (ICAM-1).

This study demonstrates that DCs infected with wild type *Y. enterocolitica* did not upregulate CD80, CD86 and CD54. Upregulation of MHC class II molecules in DCs infected with wild type *Y. enterocolitica* was also reduced compared to incubation of DCs with LPS or infection with the plasmid-cured mutant strain. This inhibited maturation of DCs observed after infection with *Y. enterocolitica* is mediated by YopP.

Similar to our observations in DCs, it was also found that *Y. pseudotuberculosis* inhibits upregulation of costimulatory molecules such as B7.2 on the surface of B cells (223). The block of B lymphocyte activation resulted from the inhibition of early phosphorylation events of the B cell receptor-signalling complex. Yao *et al*. could show, that this effect was dependent on the production of YopH, a tyrosine phosphatase (223).

Analyses of human DCs infected with *Y. enterocolitica* revealed a marked but transient down-regulation of MHC class II molecules on day 3 after infection, and down-regulation to a lesser extent for CD80 on day 5 (178). In contrast, within 24 h

infection, DCs upregulated CD83, and CD86 on day 3, which indicates maturation of DCs. One big difference to the data described herein is that the changes in surface molecule expression on human DCs were obvious 3 to 5 days post infection, whereas in mouse DCs the described inhibition of maturation was obvious 3 h post infection. As mentioned earlier, one possible explanation for these differences is the use of different *Yersinia* strains in both studies. In addition, the experiments from Schoppet *et al*. were performed using bacteria grown at 27°C over night without further incubation at 37°C. As *Y. enterocolitica* incubation at 37°C induces the production of Yops (104), only chromosomally-encoded virulence factors were acting on the DCs in the experiments performed by Schoppet *et al*.

Effects on DC maturation have also been described for other pathogens. It has been shown that *Plasmodium falciparum* (209), *Trypanosoma cruzi* (211), and herpes simplex virus (171) prevent DC maturation. Adherence of *P. falciparum*-infected erythrocytes to DCs inhibits the maturation of these DCs. In the case of *T. cruzi* an unknown soluble factor is responsible for the suppressed maturation of DCs.

In contrast, *Mycobacterium tuberculosis* (82), *Streptococcus gordonii* (44), and *Chlamydia psittaci* (139) induce activation and maturation of DCs.

DCs release a number of cytokines upon exposure to bacterial LPS (76). Cytokines are key mediators of the immune responses and their release can activate cells of the innate and adaptive immunity. For example, IL-12, IL-18 and IL-23 can promote T_H1 responses whereas IL-4 induces T_H2 responses. IL-10 induces regulatory T cells. IL-12 is also able to activate NK cells and IL-8 recruits neutrophils to the site of infection. TNF-α plays a major role in the inflammatory response by recruiting and activating monocytes and neutrophils.

In addition to the inhibited upregulation of surface molecules on DCs necessary for the formation of the immunological synapse, this study shows that *Y. enterocolitica* suppresses cytokine production, including IL-12, IL-10, TNF-α, and KC by DCs. Again, this effect is mediated via the action of YopP. These results are consistent with previously published data for mouse macrophages and epithelial cells (177,183). For mouse macrophages it was shown that YopP of *Y. enterocolitica* as well as YopJ in *Y. pseudotuberculosis* suppress TNF-α production (30,142,167). In these cases both, the inhibition of the NF-κB and the MAPK signalling pathway leads to the

suppression of TNF-α. Schulte *et al*. showed that Yops of *Y. enterocolitica* inhibit IL-8 production by human epithelial cells (181).

The temporary activation of NF-κB in DCs observed after infection with wild type *Yersinia* (pYV^+) might explain the difference between the slight upregulation of MHC class II molecules and the lack of costimulatory molecules and cytokine release. In contrast to cytokines and costimulatory molecules, preformed MHC class II molecules are abundant in intracellular compartments of non-activated DCs (96). While limited activation of DCs might be sufficient to stimulate the transport of preformed MHC class II molecules to the cell surface of DCs, *de novo* synthesis of cytokines may require a prolonged period of cell activation. Rescigno *et al*. showed that LPS induced maturation of DCs is dependent on NF-κB activation, measured by upregulation of molecules such as MHC and CD86 and TNF-α release (156). Therefore, it is likely that YopP inhibits maturation of DCs via inhibition of NF-κB.

In summary, these results indicate that YopP is the most potent pathogenicity factor of *Y. enterocolitica* that prevents DC mediated activation of $CD4^+$ T cells by induction of apoptosis, inhibition of DC maturation, and cytokine production. To examine whether additional functions of DCs like antigen uptake and processing are affected by *Y. enterocolitica*, T cell proliferation assays with Ova peptide as antigen were performed. The Ova peptide can bind directly to the MHC II molecules on the cell surface without uptake and processing. Incubation of DCs with Ova peptide after infection with wild type *Y. enterocolitica* did not lead to a reduced T cell proliferation compared to uninfected DCs. This result indicates that *Y. enterocolitica* affects somehow antigen uptake and/or antigen processing capacity of DCs. On the other hand it demonstrates that although DCs undergo apoptosis and are compromised in maturation and secretion of proinflammatory cytokines, they are able to stimulate $CD4^+$ T cells efficiently. Therefore, it is more likely that antigen uptake and/or antigen processing play the major roles in preventing T cell activation by DCs.

4.4 *Y. enterocolitica* inhibits antigen uptake and processing in DCs

Endocytosis is essential for the presentation of antigens by major histocompatiblity complex (MHC) class II molecules. In general, the endocytic capacity of DCs is high in the immature stage and down-regulated during maturation (69,200,220). DCs can capture soluble antigen mainly via two distinct mechanisms.

First, the non-specific mechanism of antigen uptake in DCs is macropinocytosis (172). Macropinocytosis is a form of high-volume, non-specific endocytosis that involves extension of membrane ruffles. Macropinocytosis is highly dependent on actin polymerisation (3) and it was shown that Rac-dependent recruitment of PAK1 kinase and its activation is both necessary and sufficient for macropinocytosis (56,57). Macropinocytosis in DCs requires Rac (69,220), and in cells derived from the bone marrow it also requires Cdc42 (69). Mellman and co-workers (69) have shown that Cdc42 is a cellular key regulator of macropinocytic function in these cells: during maturation DCs selectively downregulate Cdc42 activity, but transfection of a constitutively active Cdc42 mutant is sufficient to reactivate macropinocytosis. However, macropinocytosis in spleen-derived cells appears to be completely independent of Cdc42 (220), suggesting that the role of Cdc42 in antigen uptake depends on the type of the DCs.

Second, the clathrin-mediated endocytosis previously referred to as receptor-mediated endocytosis (43) comprising endocytosis of receptors like low-density-lipoprotein receptor (LDLR), transferrin receptor (TfR), macrophage mannose receptor (MMR), Fc receptors, and members of the C-type lectin family (59,152,172,218). These receptors localize to coated pits, which highly increase antigen uptake via clathrin-coated vesicles (CCV). In the formation of these CCVs several accessory proteins including amphiphysin, dynamin, AP2, and episin are involved. It was shown that actin-disrupting agents had only partial or no effect on the formation of CCVs (65). Contrary findings from Merrifield *et al.* revealed that actin assembly is spatially and temporally coordinated with the recruitment of dynamin to the necks of coated pits, and with the release of CCVs into the cytosol (127). These findings suggest that the actin cytoskeleton is not absolutely required for clathrin-mediated endocytosis, but instead appears to contribute to the

organization and efficiency of this process. Similarly, while Rho GTPases do not have an obligate function in clathrin-mediated endocytosis, several studies suggest that these signalling proteins act to regulate the efficiency of internalisation. For example, Schmid and co-workers showed that constitutively active mutants of either Rac or RhoA block internalisation of the transferrin receptor (103). However, expression of dominant-negative mutants of these signalling proteins had no effect on receptor uptake in intact cells, suggesting that the observed effects of constitutively active Rac and RhoA may be an indirect consequence of locking these proteins in the active form. In addition, studies with dominant-negative mutants demonstrated that Cdc42 is not generally required for clathrin-mediated endocytosis (102). While clathrin-mediated endocytosis acts constitutively in both immature and mature DCs, macropinocytosis is down-regulated in mature DCs.

In a first approach antigen uptake was analysed by flow cytometry and fluororeader using Ova-FITC as antigen because T cell proliferation assays were performed with Ova protein. It is known that Ova is taken up by macropinocytosis (65%) as well as clathrin-mediated endocytosis (35%) into a fetal skin-derived dendritic cell line representing an immature stage (116). The results observed in this study demonstrate that *Y. enterocolitica* pYV^{+}, but not the YopP-deficient mutant strain (pYV$^{+}\Delta$*yopP*), inhibits Ova-FITC uptake by 45%, suggesting that YopP is responsible for this decreased antigen uptake in DCs. Consistently, infection of DCs with mutant strains deficient for YopE, YopH, and YopT resulted in reduced Ova-FITC uptake comparable with the wild type *Y. enterocolitica* strain (data not shown). Interestingly, YopE, YopT, and YopH inhibit actin polymerisation by interacting with Rho GTPases or focal adhesion kinases. Therefore, one would expect that if Ova uptake is mainly mediated by macropinocytosis the knock out of YopE, YopT, and YopH should result in increased uptake of Ova-FITC. It is, however, possible that knock out of one Yop can be overcome by the two others, leading to the same inhibition of Ova uptake observed with the wild type *Yersinia*. A second possibility would be that Ova uptake in mouse bone marrow derived DCs is mediated predominantly via clathrin-mediated endocytosis.

YopP is known to inhibit the NF-κB and the MAPK pathways (140). Using inhibitors of both pathways we could show that inhibition of the MAPK pathway led to a reduction

of Ova uptake comparable to that observed with DCs infected with wild type *Yersinia*. In contrast to this, several inhibitors of the NF-κB signalling pathway did not influence Ova uptake in DCs (data not shown). Therefore it is likely that YopP inhibits Ova uptake via the inhibition of MAPKs. To confirm this hypothesis the inhibitory effect of YopP on MAPKs was mimicked by infecting DCs with the YopP-deficient mutant strain and subsequently adding MAPK inhibitors. The results demonstrate that the addition of MAPK inhibitors to DCs infected with *Y. enterocolitica* pYV$^{+}\Delta$*yopP* leads to a reduction of antigen uptake; thus, the inhibitors "complemented" for the YopP deficiency.

To point out a direct link between the involvement of MAPKs in endocytosis, transfection experiments in DCs were performed to over-express dominant negative and wild type (controls) constructs for JNK2 and p38 (99). To confirm the functionality of the constructs in transfected DCs western blot analyses were performed using antibodies to phosphorylated and non-phosphorylated MAPKs. Western blot analyses of p38 expression demonstrated an over-expression of p38 in DCs transfected with both, the dominant negative and wild type constructs compared to the mock control. Indeed, phosphorylation of p38 was high in DCs transfected with the wild type control for p38, but not with the dominant negative construct (data not shown). These data demonstrate the successful transfection and the functionality of the dominant negative construct for p38. Comparable results were obtained with the constructs for JNK2 except for the fact that the phosphorylation of JNK was not inhibited with the dominant negative construct, which can be explained by the fact that the phosphorylation-binding site is conserved in this dominant negative mutant for JNK (M. Kracht, personal communication). Therefore, the p-JNK antibody is able to bind to the over expressed protein. The dominant negative mutant inactivates the kinase by a mutation in their ATP binding site.

Preliminary results from these studies revealed no differences in Ova uptake between DCs transfected with the dominant negative and the wild type constructs for p38, JNK, or a mixture of the two constructs. It is likely that despite transfection endogenous MAPKs get still phosphorylated. Alternatively the effect of JNK may not depend on the enzymatic activity of the protein, but depend on protein-protein interactions, that might not be altered by mutation of JNK (M. Kracht, personal

communication). To overcome this problem siRNA transfections are necessary to prove that MAPKs are involved in antigen uptake.

As DCs may take up Ova by both macropinocytosis and clathrin-mediated endocytosis we addressed which of these pathways is involved in our system by using inhibitors of each pathway. Competition of mannan with Ova-FITC reduced Ova uptake up to15 % indicating a minor role for the MMR in Ova uptake. In contrast, Ova uptake was highly actin dependent, as incubation of DCs with CyD, which blocks actin polymerisation, reduced Ova uptake up to 55%. These results are in agreement with the findings that macropinocytosis as well as clathrin-mediated endocytosis are actin dependent, the latter to a lesser degree (3,127). As co-incubation of DCs with *Yersinia* wild type strain and CyD synergistically reduced Ova uptake one can conclude that inhibition of Ova uptake by *Yersinia* is partially independent of actin assembly. As macropinocytosis is highly dependent on Rho GTPases toxin B from *C. difficile* was added, which inhibits RhoA, Rac1, and Cdc42 prior to addition of Ova. We observed no significant reduction of Ova uptake by toxin B from *C. difficile*, indicating a minor role for Rho GTPases in Ova uptake. These data are in contrast to recent findings from Mellman and co-workers, demonstrating that toxin B from *C. difficile* inhibits uptake of Ova up to 80% (69). In the study from Mellman and co-workers DCs were generated from C3H/HeN mice according to the protocol of Inaba *et al.* (88) and used for experiments at day 6 of culture. In contrast, the DCs used in this study were from BALB/c mice according to the protocol of Lutz *et al.* (115) and the cells were used for the experiments at day 8 of culture. Therefore, we generated DCs according to both protocols and analysed their maturation stage, as well as Ova uptake with or without addition of TcdB (data not shown). Preliminary data show that the role of Rho GTPases in Ova-FITC uptake by DCs depends on mouse strains.

The experiments performed with the inhibitors of proteins involved in endocytosis did not clarify exactly to what degree Ova is taken up by macropinocytosis or clathrin-mediated endocytosis. This is due to the fact that actin is involved in both macropinocytosis as well as clathrin-mediated endocytosis.

Therefore, Tf and LY, which are internalised by molecular defined pathways, were used to further address this issue. Tf was used as a marker for clathrin-mediated

endocytosis, because it is specifically internalised via binding to the Tf-receptor (116). Clathrin-mediated endocytosis of Tf-TfR complex is a constitutive process. The complex is taken up into early endosomes, where Tf is released from the receptor, which is rapidly recycled to the cell surface (42). Both *Y. enterocolitica* wild type and MAPK inhibitors reduced uptake of Tf in DCs whereas the YopP-deficient *Yersinia* mutant strain did not. These results correspond to those obtained with Ova and demonstrate that YopP of *Y. enterocolitica* reduces clathrin-mediated endocytosis most likely by inhibiting MAPKs.

Interestingly, macropinocytosis of LY in DCs infected with *Y. enterocolitica* was also inhibited up to 70%, indicating that *Y. enterocolitica* is also able to block macropinocytosis. However, uptake of LY by DCs infected with the YopP-deficient mutant strain was reduced comparable to the *Yersinia* wild type strain. These data demonstrate that YopP does not play a role in the inhibition of macropinocytosis in DCs infected with *Y. enterocolitica*. In agreement with these findings, incubation of DCs with MAPK inhibitors did not significantly reduce uptake of LY. In contrast, TcdB and CyD dramatically reduced LY uptake in DCs. Based on these results it is more likely that YopE, YopH, or YopT are involved in the inhibition of macropinocytosis in DCs by *Y. enterocolitica*. To find out which of these Yops affects macropinocytosis in DCs further experiments using mutant strains for YopE, YopH, and YopT are needed.

Taken together, the data observed with Tf and LY demonstrate that YopP of *Y. enterocolitica* inhibits clathrin-mediated endocytosis of Tf, but not macropinocytosis of LY. As inhibition of Rho GTPases reduced uptake of LY in DCs, but not uptake of Ova, one can conclude that Ova is endocytosed by DCs from BALB/c mice according to the protocol of Lutz *et al*. mainly via clathrin-mediated endocytosis.

A well-documented characteristic of DC maturation is the progressive down-regulation of endocytosis (69,172,220). Recently Watts and co-workers demonstrated that TLR ligands first acutely stimulate antigen macropinocytosis (221). In this study they stimulated DCs with low doses of LPS for 30 min and then pulsed with FITC-dextran for 10 min and analysed for antigen uptake. They observed several times more accumulation of FITC-dextran in DCs with LPS stimulation compared to untreated DCs. However, this enhancement was transient, peaking after 30 min of LPS stimulation in bone marrow DCs. This transient upregulation of

endocytosis was dependent on actin assembly and p38 and ERK activation. To assess whether in the system used in this study the observed inhibition of Ova uptake in DCs treated with *Y. enterocolitica* and/or MAPK inhibitors is due to the inhibition of LPS stimulated MAPKs, DCs from $TLR2^{-/-}xTLR4^{-/-}$ mice were infected with *Yersinia* wild type and the YopP-deficient mutant strain, as well as treated with MAPK inhibitors and analysed for Ova uptake. The results revealed that Ova uptake by DCs infected with *Y. enterocolitica* or incubated with inhibitors of MAPKs is independent of the TLR2 and TLR4 signalling pathway, indicating a differential role for MAPKs than that described by West *et al.* (221). Similar results were also obtained for DCs from $MyD88^{-/-}$ and $TRIF^{-/-}$ mice.

Little is known about the role of MAPKs in endocytosis. Recent findings from Shang *et al.* revealed an important role for MAPKs in the regulation of amphiphysin 1 during clathrin-mediated endocytosis of the NGF receptor (187). Amphiphysin 1, which can simultaneously bind to dynamin 1 and the clathrin adaptor AP-2, is essential for dynamin recruitment during clathrin-mediated endocytosis (Fig. 4.1). The authors found that amphiphysin 1 is phophorylated by MAPKs in response to NGF stimulation of PC12 cells. MAPK-dependent phosphorylation of amphiphysin 1 negatively regulated the association of amphiphysin1 with AP-2 adaptor. Therefore, they speculate that MAPKs control NGF receptor-mediated endocytosis by terminating the interaction between amphiphysin1 and AP-2. In contrast to our findings, the results from Shang *et al.* demonstrate that activation of MAPKs inhibits clathrin-mediated endocytosis.

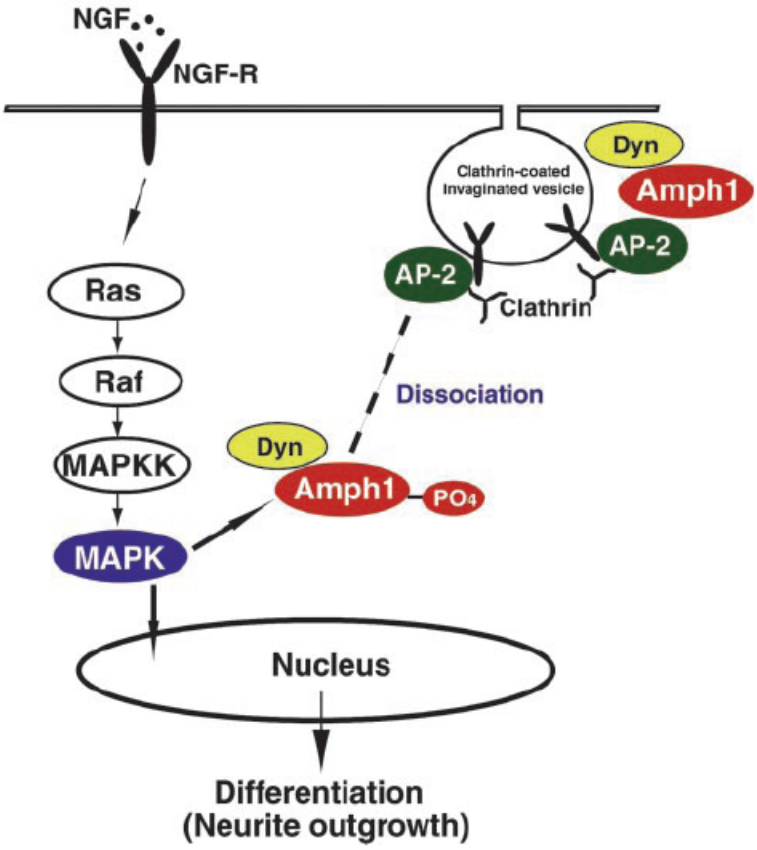

Fig. 4.1: **Model for the role of MAPKs in phosphorylation of amphiphysin (from Shang *et al.* (187))**
NGF stimulates the NGF receptor tyrosine kinase TrkA and in turn transmits activation signals through the Ras-Raf-MAPKKMAPK pathway to the nucleus, leading to neurite outgrowth in PC12 cells. Concomitantly, MAPKs phosphorylate amphiphysin1, and phosphorylated amphiphysin1 dissociates from the AP-2 adaptor, resulting in the recycling of amphiphysin1-dynamin (Dyn) complexes during NGF receptor-mediated endocytosis.

A third study reported that the MAPK p38 regulates endocytic trafficking via the GDI:Rab5 complex (36). Rab5 is one of the key regulators of early endocytic traffic and coordinates different trafficking events, like homotypic early endosome fusion (70), internalisation (34), clathrin-coated vesicle formation (124), and motility of early endosomes on microtubules (136). Rab5 cycles between GTP- and GDP-bound states as well as between membrane and cytosol. Cavalli *et al.* provide evidence that the protein kinase p38 MAPK regulates GDI activity in the cytosolic cycle of the small GTPase Rab5 (36). They also found that activation of p38 MAPK modulates endocytic rates, and this modulation was reduced in p38$\alpha^{-/-}$ fibroblasts. These data indicate that membrane traffic can be controlled by external stimuli, emphasizing the possible role of trafficking regulation in infection, aging, and a number of degenerative

diseases. Therefore, we speculate that inhibition of endocytosis by YopP via MAPKs may lead to a reduced formation of GDI:Rab5 complexes, which needs to be investigated in future studies.

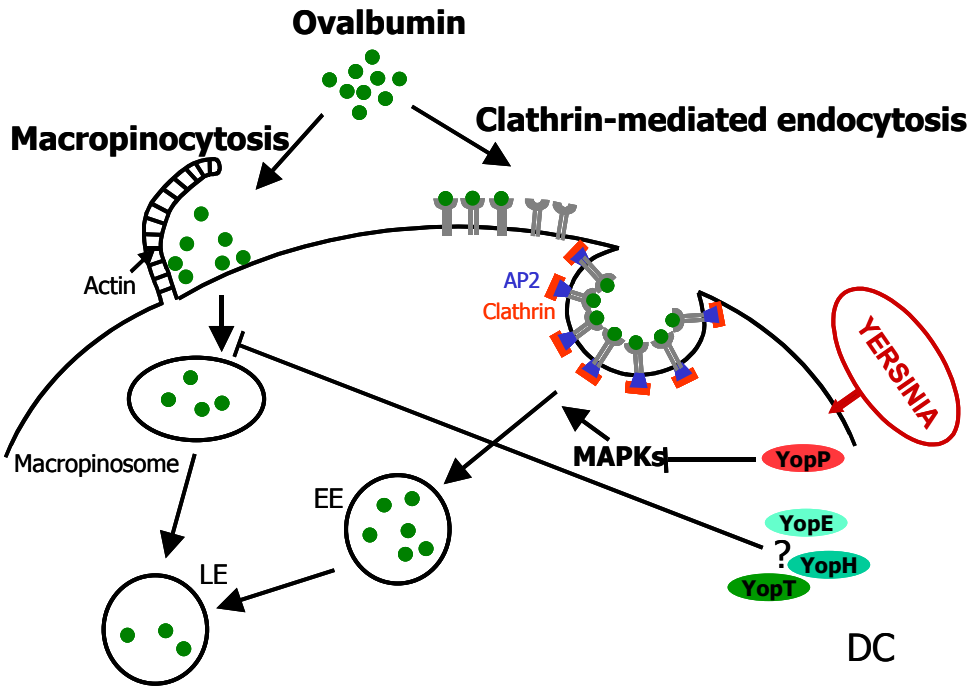

Fig. 4.2: Model for the modulation of endocytosis by *Y. enterocolitica*
Y. enterocolitica inhibits clathrin-mediated endocytosis as well as macropinocytosis. Inhibition of clathrin-mediated endocytosis by *Yersinia* is mediated by YopP most likely via the inhibition of MAPKs. Inhibition of macropinocytosis is independent of YopP. EE early endosome; LE late endosome

Taken together, the studies reported in this thesis demonstrate that YopP of *Y. enterocolitica* is able to inhibit antigen uptake via clathrin-mediated endocytosis most likely by inhibiting MAPK signalling pathways (Fig. 4.2). Macropinocytosis in DCs is also inhibited by *Yersinia*, but until now it is not clear which effector protein is responsible for this effect. Therefore, further studies including siRNA and co-immunoprecipitation experiments are needed to build a direct link between YopP and MAPKs and to elucidate, which of the proteins that are essential in endocytosis are targeted by MAPKs in DCs by performing, respectively.

In addition, we found that *Y. enterocolitica* inhibits the activation of proteins essential for antigen degradation and maturation of newly synthesized MHC class II

molecules (85). The results reveal that the inhibition is not selective for one cathepsin and the amount of total cathepsins is not affected by *Y. enterocolitica*. The inhibition of cathepsins is dependent on plasmid-encoded factors, but not on YopP. Experiments using mutant strains deficient for single Yops did not clarify which effector molecule of *Y. enterocolitica* prevent the activation of the cathepsins. Therefore, it is possible that two or more Yops synergistically inhibit the activation of cathepsins. These results indicate that *Y. enterocolitica* may affect antigen processing in DCs. Whether the inhibition of cathepsins by *Y. enterocolitica* in DCs leads to inhibition of antigen degradation and MHC class II maturation needs to be analysed in further experiments.

Molinari *et al.* demonstrated that *Helicobacter pylori* inhibits antigen processing in a B cell line (131). By using T cell clones with different specificity, they found that the toxin VacA of *H. pylori* interferes with the generation of T cell epitopes loaded on newly synthesized molecules (the invariant chain [Ii]-dependent pathway of antigen presentation), leaving unaffected generation and presentation of epitopes by class II molecules that recycle through early endosomal compartments (Ii-independent pathway).

In conclusion, this study demonstrates that plasmid-encoded virulence factors of *Y. enterocolitica* impair the T cell activation capacity of mouse DCs by various means. YopP has found to be the most prominent effector protein of *Y. enterocolitica* affecting immunological functions of DCs (Fig. 4.3). YopP induces cell death in DCs by a mechanism that has features of both apoptosis and necrosis. The mechanism as well as the target proteins of YopP involved in the induction of cell death still have to be investigated. By its ability to inhibit NF-κB activation, YopP blocks the upregulation of MHC class II and costimulatory molecules, which requires NF-κB activation (156). YopP of *Y. enterocolitica* inhibits the production of proinflammatory cytokines and cytokines required for T cell activation and differentiation into effector T cells, which is dependent on activation of NF-κB and MAPK pathways (156). Furthermore, YopP reduces most likely via inhibition of MAPKs, antigen uptake by clathrin-mediated endocytosis, whereas macropinocytosis is reduced by other yet not defined effector proteins. It would be interesting to analyse the role of MAPKs in clathrin-mediated endocytosis and their link to YopP. In addition, inhibited activation

of cathepsins in DCs infected with different mutant strains of *Y. enterocolitica* provided evidence that antigen processing in DCs is also impaired by *Y. enterocolitica*. However, it is not clear whether this effect is also mediated by YopP or by other effector proteins. Furthermore, it has to be investigated which part of the antigen processing pathway may be affected by the inhibited activation of cathepsins caused by *Y. enterocolitica* in DCs. However, as these observations have been obtained by *in vitro* experiments, therefore further studies are required in order to investigate whether the aforementioned functions of DCs affected by *Y. enterocolitica* might subvert the adaptive immune defence mechanisms of the host *in vivo*.

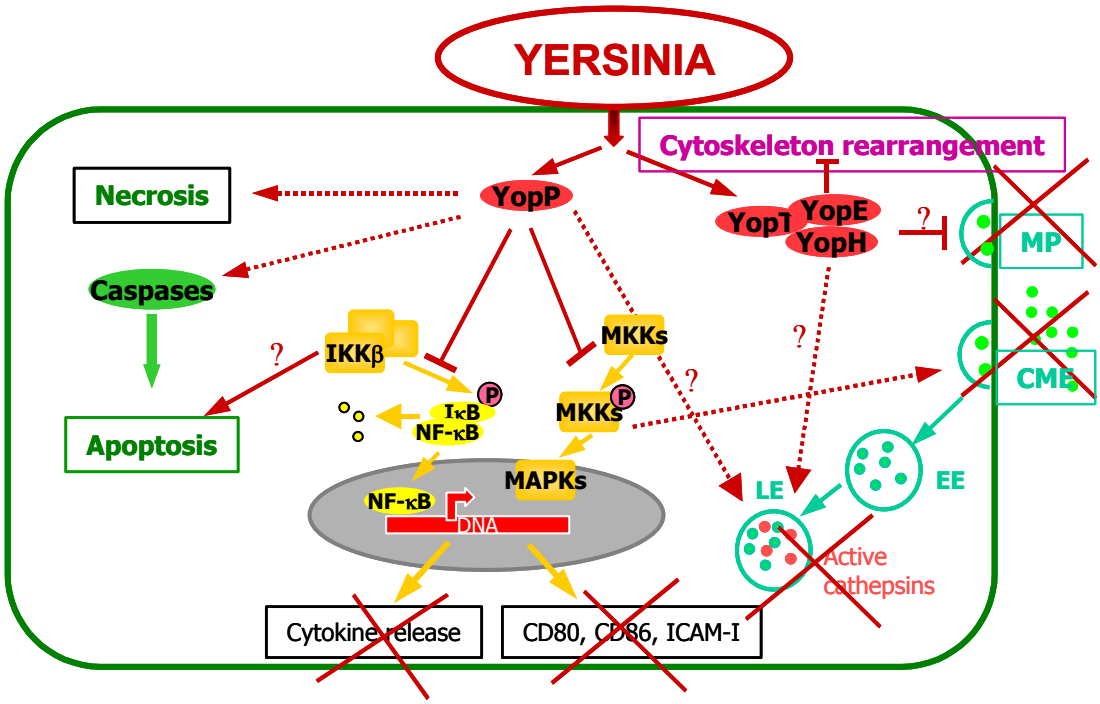

Fig. 4.3: Model for the modulation of DCs by *Y. enterocolitica*; adapted from Cornelis *et al.* (46)

5 Summary

Yersinia enterocolitica is able to evade components of the innate immune system like phagocytosis by PMNs and lysis by complement by proteins encoded by the virulence plasmid pYV like the outer membrane protein YadA and secreted anti-host effector proteins (Yops). The production of the cytokines TNF-α, IL-12, IL-18, and IFN-γ as well as the activation of CD4 T cells and macrophages are essential to control *Yersinia* infection. Whether *Y. enterocolitica* also affects adaptive immune responses was not yet known. The aim of this work was to investigate whether and how *Y. enterocolitica* affects the immunogenic properties of mouse DCs *in vitro*. The results demonstrate that plasmid-encoded pathogenicity factors of *Y. enterocolitica* completely abolish the ability of DCs to activated $CD4^+$ T cells. Taking advantage of *Y. enterocolitica* mutant strains deficient for various Yops, it was demonstrated that particularly the secreted protein YopP accomplished the immune evasion of DCs by various effects: (i) *Y. enterocolitica* YopP induces cell death in mouse DCs. (ii) YopP inhibits maturation of DCs as demonstrated by reduced upregulation of MHC class II and costimulatory molecules and inhibition of cytokine release. (iii) *Y. enterocolitica* reduces antigen uptake in DCs by two different mechanisms: YopP inhibits clathrin-mediated endocytosis, most likely via the inhibition of MAPK; in addition, other not yet defined effector proteins of *Y. enterocolitica* reduce macropinocytosis. (iv) *Y. enterocolitica* also inhibits the activation of cathepsins, molecules involved in antigen degradation and MHC class II maturation, suggesting that effector proteins of *Y. enterocolitica* affect antigen processing in DCs. These results demonstrate that YopP is a multifunctional effector protein, which disturbs several cell functions required to mount adaptive immune responses. Moreover, the results show that analyses of molecular basis of microbial virulence factors reveal interesting insights into host cell functions and immune responses. Further studies will have to reveal the target proteins and mechanisms involved in YopP mediated cell death and inhibition of clathrin-mediated endocytosis. As these observations have been obtained by *in vitro* experiments, further studies will also have to elucidate whether by targeting the aforementioned functions of DCs *Y. enterocolitica* might actually contribute to subvert the adaptive immune defence mechanisms of the host *in vivo*.

6 Abbreviations

Ail	attachment invasion locus
Amp^R	ampicilin resistant
APC	antigen presenting cell
APS	ammoniumpersulfat
BCA	bicinchoninic acid
BSA	bovine serum albumin
C	carbon
Ca	Calcium
Cat	cathepsin
CCR	chemokine receptor
CD	cluster of differentiation
CFSE	5-(6)-carboxyfluoresceine diacetate N-succinimidylester (CFDA,SE)
Cl	Chlor
CLIP	class II-associated invariant chain peptide
Cm^R	Chloramphenicol-Resistenz
CME	clathrin-mediated endocytosis
CR	complement receptor
CTL	cytotoxic T lymphocyte
CyD	cytochalasin D
DC	dendritic cell
DMSO	Dimethylsulfoxid
DNA	desoxyribonucleic acid
dNTP	Desoxyribonucleosidtriphosphate
dTTP	desoxy-thymidintriphosphate
ECL	enhanced chemoluminescence
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmatic Reticulum
et al.	and others
FACS	flourescence activated cell sorter
Fig.	figure
FITC	fluoresceine isothiocyanate
FCS	fetal calf serum
G	guanine
g	Earth's acceleration
GALT	gut associated lymphoid tissue
GM-CSF	granulocyte-monocyte colony stimulating factor
H	hydrogen
h	hour
HBSS	Hank's balanced salt solution
HK	heat-killed
HLA	human leucocyte antigen
HPI	high pathogenicity island
HRP	horse-radish peroxidase
IFN	interferon
Ig	immunglobulin
Ii	invariant chain
IL	interleukin
Inv	invasin
i.v.	intravenously
kDa	kilo Dalton
LB	Luria Bertani
Lcr	low Calcium response
LPS	lipopolysaccharid
LY	lucifer yellow
MCP-1	macrophage chemoattractant protein
M	molar
MALT	mucosa associated lymphoid tissue
MAPK	mitogen activated protein kinase
Mg	magnesium
MHC	major histocompatibility complex
min	minute
MOI	multiplicity of infection
Myf	mucoid Yersinia factor
M cell	membraneous cell
N	nitrogen
Na	sodium
Nal^R	nalidixin resistant
NF	nuclear factor
NK	natural killer
O	oxygen
OD	optical density
Ova	Ovalbumin
P	Phosphor
p	probability
PAGE	polyacrylamide gel electrophoresis
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered saline
PE	Phycoerythrin
PFA	paraformaldehyde
PI	propidium iodide
PMN	polymorphonuclear neutrophils
PMSF	phenyl-methyl-sulfonylfluoride
pYV	plasmid *Yersinia* Virulence
PVDF	polyvinylidenfluoride
Rho	Ras homologous
RNA	ribonucleic acid
rpm	rounds per minute
RPMI	Roswell Park Memorial Institute
SD	standard deviation
SDS	sodium dodecyl sulfate
sodA	superoxid dismutase A
$Spec^R$	spectinomycin resistant
ssp.	species
Syc	specific Yop chaperone
T	thymidine
Tab.	table
TAP	transporter associated with antigen processing
TcdB	*Clostridium difficile* toxin B
TCR	T cell receptor
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
Tf	transferrin
T_H1	T helper cell type 1
T_H2	T helper cell type 2
TLR	Toll like receptor
Tris	tris(hydroxymethyl)aminomethan
TTSS	type three secretion system
IKK	inducible IκB-kinase
TNF	Tumor necrosis factor
U	unit
YadA	*Yersinia* adhesin A
Yop	*Yersinia* outer protein

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8 Publications

S. E. Erfurth, S. Gröbner, U. Kramer, D. S. J. Gunst, I. Soldanova, M. Schaller, I. B. Autenrieth, S. Borgmann

Yersinia enterocolitica induces apoptosis and inhibits surface molecule expression and cytokine production in murine dendritic cells.

Infect Immun. 2004 Dec;72(12):7045-54.

Danksagung

Bei Herrn Prof. Dr. I. B. Autenrieth bedanke ich mich ganz herzlich für die interessante Themenstellung, für seine ständige Bereitschaft zur Diskussion und Beratung, sowie seine kontinuierliche Unterstützung.

Bei Dr. Stefan Borgmann bedanke ich mich für die aktive Betreuung, die Einführung in die Welt der DCs und T-Zellen, sowie für seine guten Ideen.

Weiterhin danke ich Prof. Dr. A. Peschel und Prof. Dr. H.-G. Rammensee für ihr Interesse an meiner Arbeit, ihre guten Ratschläge und ihre Bereitschaft, diese Dissertation vor der Fakultät für Chemie und Pharmazie der Eberhard Karls Universität Tübingen zu vertreten.

Für ihre Kooperationsbereitschaft und Unterstützung, ohne die Teile dieser Arbeit nicht möglich gewesen wären, möchte ich mich bei Herrn Prof. Dr. M. Kracht, Herrn Prof. Dr. M. Schaller, Herrn Dr. Ch. Driessen bedanken.

Mein besonderer Dank gilt Daniela Gunst, Roman Rösemann und Michael Reich, ohne die die Bearbeitung dieses Themas nie so erfolgreich verlaufen wäre.
Bei Herrn Dr. Erwin Bohn möchte ich mich für seine Hilfsbereitschaft und Ratschläge bedanken.
Bedanken möchte ich mich auch herzlich bei Edda Fahl, Susanne Berchtold, Martin Köberle, Gianluca Matteoli, Irena Soldanova für das Korrekturlesen meiner Doktorarbeit und bei Birgit Manncke, Juliane Krejci, Isabell Wenzel, Guntram Grassl, Uwe Kramer, Carolin Wiedig, Naima Zahir, Susanne Berchtold und Martin Köberle für ihre ständige Bereitschaft meine Fragen zu beantworten und mir bei allen Problemen Hilfestellung zu leisten, sowie bei Tamara Meinhardt-Krajina für die guten Tipps beim FACSen.
Außerdem möchte ich mich bei allen Mitarbeitern des Instituts für die angenehme Zeit im Kaffeeraum bzw. im Labor bedanken und für das sehr gute Arbeitsklima, das ein angenehmes und erfolgreiches Forschen ermöglichte.

Der allergrößte Dank gebührt meinen Eltern, die mich immer unterstützt und in meinen Entscheidungen bestärkt haben.

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