Nadzeya Kramko

Molecular interactions of enteropathogenic and probiotic bacteria with cells involved in the surveillance of the gastrointestinal barrier

-2009-

Biologie

Molecular interactions of enteropathogenic and probiotic bacteria with cells involved in the surveillance of the gastrointestinal barrier

Inaugural-Dissertation
zur Erlangung des Doktorgrades
der Naturwissenschaften im Fachbereich Biologie
der Mathematisch-Naturwissenschaftlichen Fakultät
der Westfälischen Wilhelms-Universität Münster

vorgelegt von
Nadzeya Kramko
aus Minsk

- 2009 -

Dekan:	Prof. Dr. Christian Klämbt
Erster Gutachter:	Prof. Dr. M. Alexander Schmidt
Zweiter Gutachter:	Prof. Dr. Volker Gerke
Tag der mündlichen Prüfung:	25.1.10
Tag der Promotion:	5.2.10

Table of contents

Abbreviations

AAF	aggregative adherence fimbria
A/E	attaching and effacing
ATEC	atypical enteropathogenic *E. coli*
ATP	adenosine triphosphate
BFP	bundle-forming pilus
BSA	bovine serum albumin
CFA	colonization factor antigen
CFSE	carboxyfluorescein succinimydil esther
CSR	class switch recombination
DAEC	diffusely adhering *E. coli*
DAF	decay accelerating factor
DAPI	4,6-Diamidino-2-phenylindol
DC	dendritic cells
ddH_2O	double-distilled water
DNA	desoxyribonucleic acid
dNTPs	desoxynucleotides triphosphate
EAEC	enteroaggregative *E. coli*
ECM	extracellular matrix
EcN	*E. coli* Nissle 1917
EHEC	enterohemorrhagic *E. coli*
EIEC	enteroinvasive *E. coli*
EPEC	enteropathogenic *E. coli*
ETEC	enterotoxigenic *E. coli*
FC	flow cytometry

FCS	fetal calf serum
FITC	fluorescein isothiocyanate
GALT	gut-associated lymphoid tissue
GM-CSF	granulocyte-macrophage-colony stimulating factor
HBMEC	human brain microvascular endothelium cells
IEC(s)	intestinal epithelial cell(s)
IF	immunofluorescence
IFR	interfollicular T cell areas
IgA	immunoglobulin A
IL	interleukin
IPTG	isopropyl-β-D-thiogalactopyranosid
LEE	locus of enterocyte effacement
LPS	lipopolysacharide
LDH	lactate dehydrogenase
miRNA	micro-RNA
MOI	multiplicity of infection
NEAA	non-essential amino acids
OD	optical density
OSM	oncostatin M
PAI	pathogenicity island
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PE	phycoerythrin
PF	paraformaldehyde
PI	propidium iodide
PMN	polymorphonuclear cell

PRR	pattern recognition receptor
RNA	ribonucleic acid
RT	room temperature
RT-PCR	real time polymerase chain reaction
SED	subepithelial dome
SEM	scanning electron microscopy
TGF	tumor growth factor
TJ	tight junction
TLR	Toll-like receptor
TNF	tumor necrosis factor
TSLP	thymic stromal lymphopoietin
TTSS	type III secretion system
Stx	Shiga toxin

1. Introduction

1.1. Common themes in bacterial virulence

Presently approximately $9x10^3$ bacterial species have been identified and partially characterized which represent only a tiny fraction of the $10^7 – 10^9$ bacterial species that have been estimated to exist. Among them are free-living organisms that can be found in almost any environment, from the Antarctic ice to the deep-sea hydrothermal vents. Many bacteria have built a mutualistic relationship with eukaryotic organisms, generating a microflora that is specifically adapted to and special to the individual organism. In some cases these interactions are so important that deprivation of its microflora is lethal for the host. Other bacteria have developed survival strategies that involve the exploitation of resources of the host, and others might even be pathogenic in causing a plethora of different diseases in plants and animals. At first look an answer to the question 'what makes a pathogen' might appear rather simple as pathogenicity was defined more than 40 years ago as 'the biochemical mechanisms whereby microorganisms cause disease' (Smith *et al*., 1968). However, in closer investigation it becomes apparent that answering this question is indeed quite complex. Many bacteria can live as commensals and activate their virulence strategies only if host defense systems are weakened, becoming opportunistic pathogens. For example, it is known that *Staphylococcus aureus* is a normal resident of human skin and the nasal cavities of many individuals where it normally causes no harm (Hu *et al*., 1995). However, given permissive circumstances e.g. conditions that compromise the host's immune system, such as an organ transplant or chronic or acquired immunodeficiencies this bacterium becomes a devastating pathogen that is particularly feared in hospitals. It is generally accepted that pathogen differs from non-pathogens by the presence of virulence factors. A virulence factor is defined as a determinant that causes damage to the host cell, or, in a broader sense, a determinant required for the survival of the pathogen in the host (Wren, 2000). Generally, there are few main tasks a pathogen has to accomplish for being

successful: to multiply, to adhere well, to reach the tissue or organ of destination, to establish infection (Finlay and Falkow, 1997). Accomplishing these tasks at one or another stage involves undermining the host's immune system.

Determinants needed for adhesion are denoted virulence factors in a broader sense, as members of the normal microflora also need to adhere well to the host surfaces and in many cases similar mechanisms are used. 'Adhesins' can be grouped into pili (fimbrial) and non-pilus (afimbrial) adhesins. Pili are fimbrial surface organelles (appendages) that differ by structure and assembly pathway. Some of the pili types are shared by a great number of pathogens, and others are typical for specific strains or species. Afimbrial adhesins vary widely in their structure and specificity to certain eukaryotic (glyco)proteins (Soto and Hultgren, 1999).

Many pathogens are invasive, that is they are able to enter certain type of cells and may often use them as a protected environment for replication and persistence. Some bacteria are promoting their phagocytosis by normally non-phagocytic cells such as epithelial or endothelial cells. Other bacteria are able to survive even inside phagocytes, which might actually help to further disseminate the pathogens in the host organism (Finlay and Falkow, 1997).

For further establishment of infectious diseases pathogenic bacteria employ various strategies, commonly also including mechanisms to suppress or subvert the host's immune system. *Salmonella typhimurium* and *Shigella flexneri*, for example, are known to cause the transmigration of PMNs to the apical side of the gastrointestinal barrier, which correlates with the ability to cause a disease. *Yersinia enterocolitica* is suppressing TNFα, which is one of the key players triggering inflammatory responses (Finlay and Falkow, 1997).

With such a large quantity and diversity of virulence factors it came as a surprise that the encoding genes are often clustered in distinct genomic regions now referred to as pathogenicity islands (PAI) that can also be found on a (virulence) plasmid. It is commonly assumed that these specific genomic regions or PAIs have been acquired by horizontal transfer. PAIs exhibit a different G+C content, are flanked by direct repeats or insertion

sequences, and may harbor also tRNA genes or phage genome fragments at their borders (Winstanley and Hart, 2001; Gophna *et al*., 2003).

1.2. Diarrhoeagenic *E. coli*

1.2.1. Virulence of enteropathogenic *E. coli*

Diarrhoeagenic *E. coli* represent a group of food borne pathogens that can cause severe diarrhoea that is especially threatening for young children (Nataro and Kaper, 1998). Diarrhoea is responsible for approximately 2 million deaths annually (Ochoa *et al*., 2008; Bryce *et al*., 2005), mostly in developing countries. Based on their specific phenotype and the expression of characteristic virulence factor profiles diarrhoeagenic *E. coli* are currently divided into 7 groups (Kaper and Nataro, 2004; Gärtner and Schmidt, 2004). Enteropathogenic *E. coli* (EPEC) form microcolonies and are considered non-invasive. Enteroinvasive *E .coli* (EIEC) are able to invade epithelial cells and spread from cell to cell in epithelial layers (Lan *et al*., 2004; Resta-Lennert and Barrett, 2002). Enteroaggregative *E. coli* (EAEC) are able to generate and adhere in biofilms (Mohamed *et al*., 2007; Mohammed *et al*., 2004). Enterotoxigenic *E. coli* (ETEC) produce several toxins (thermostable and thermolabile enterotoxins). The thermolabile toxin (LT) was shown to be delivered into epithelial cells in vesicles (Qadri, 2005; Lasaro *et al*., 2008; Johnson *et al*., 2009; Roy *et al*., 2009). Enterohemorrhagic *E. coli* (EHEC) secrete Shiga toxins, are often associated with bloody diarrhoea, and can cause severe complications such as the hemolytic uremic syndrome (HUS) (Thorpe *et al*., 2004; Ogura *et al*., 2007). Atypical enteropathogenic *E. coli* (ATEC) are closely related to EPEC and EHEC, but cannot be included in neither of these groups as they do not express Shiga toxins and do not harbor the EAF plasmid of EPEC strains (Gärtner and Schmidt, 2004; Müller *et al*., 2009). Diffusely adhering E. coli (DAEC) are associated with urinary tract and enteric infections and supposedly evolved from intestinal commensal strains (Betis *et al*., 2003; Brest *et al*., 2004; Servin, 2005). A representation of their characteristic histopathological effects on epithelial cell can be seen in Fig.1.

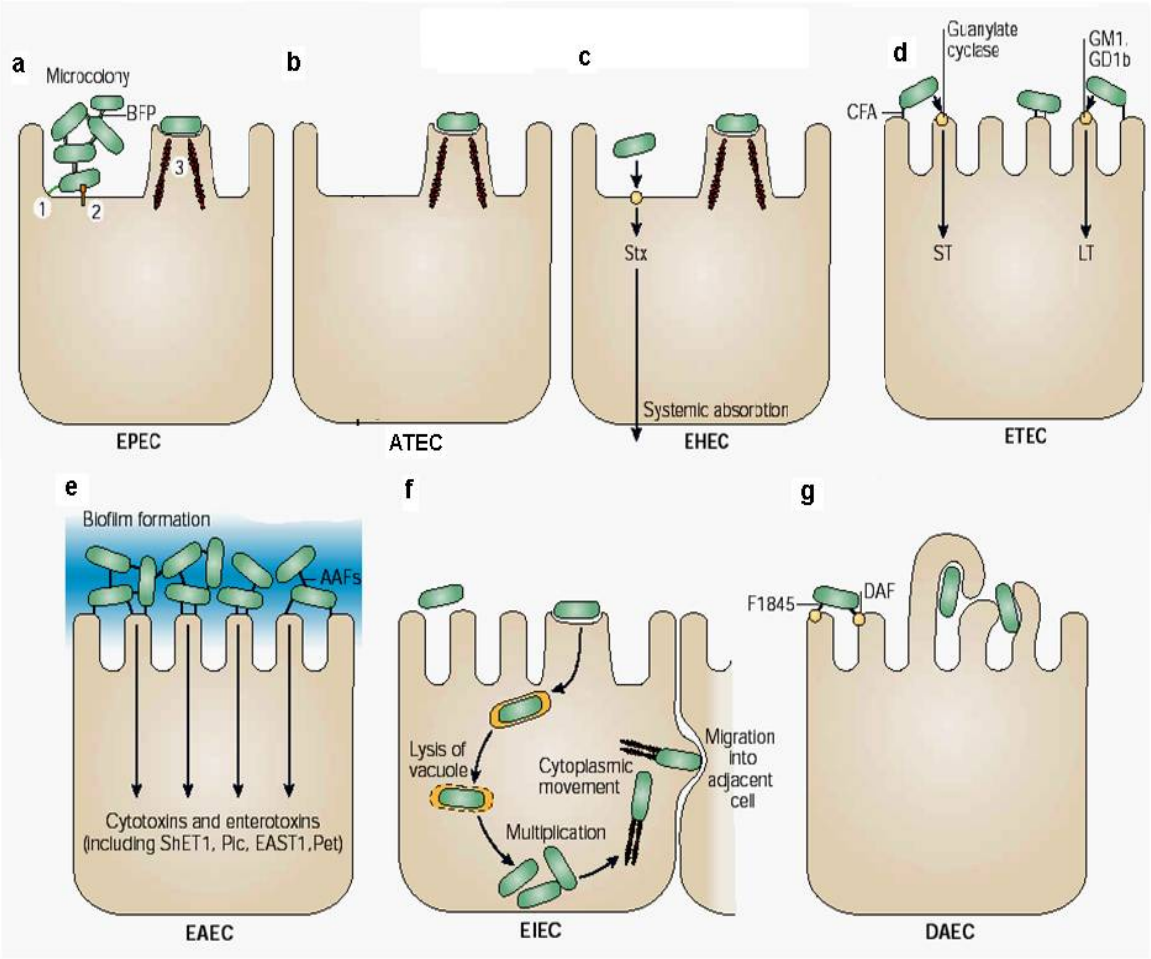


Fig. 1. The seven recognized types of diarrhoeagenic *E. coli* and their interactions with epithelial cells. BFP – bundle-forming pilus; Stx – Shiga toxin; CFA – colonization factor antigen; GM1 and GD1b – host surface glycosphingolipids; ST – heat stable toxin; LT – heat labile toxin; AAFs – aggregative adherence fimbriae; F1845 – fimbrial adhesin 1845; DAF – decay accelerating factor Modified according to Kaper and Nataro, 2004.

1.2.2. Attaching and effacing (A/E) pathogens

EPEC, ATEC, and EHEC belong to the so-called attaching and effacing (A/E) pathogens. This group of pathogens, that also includes the murine pathogen *Citrobacter rodentium* is characterized by the formation of A/E lesions on infected intestinal layer. A/E lesions are characterized by local microvilli destruction and the formation of actin-rich protrusions

called 'pedestals' just underneath the site of bacterial attachment. At the same time microvilli adjacent to the lesion are elongated (Nataro and Kaper, 1998) (Fig.2).

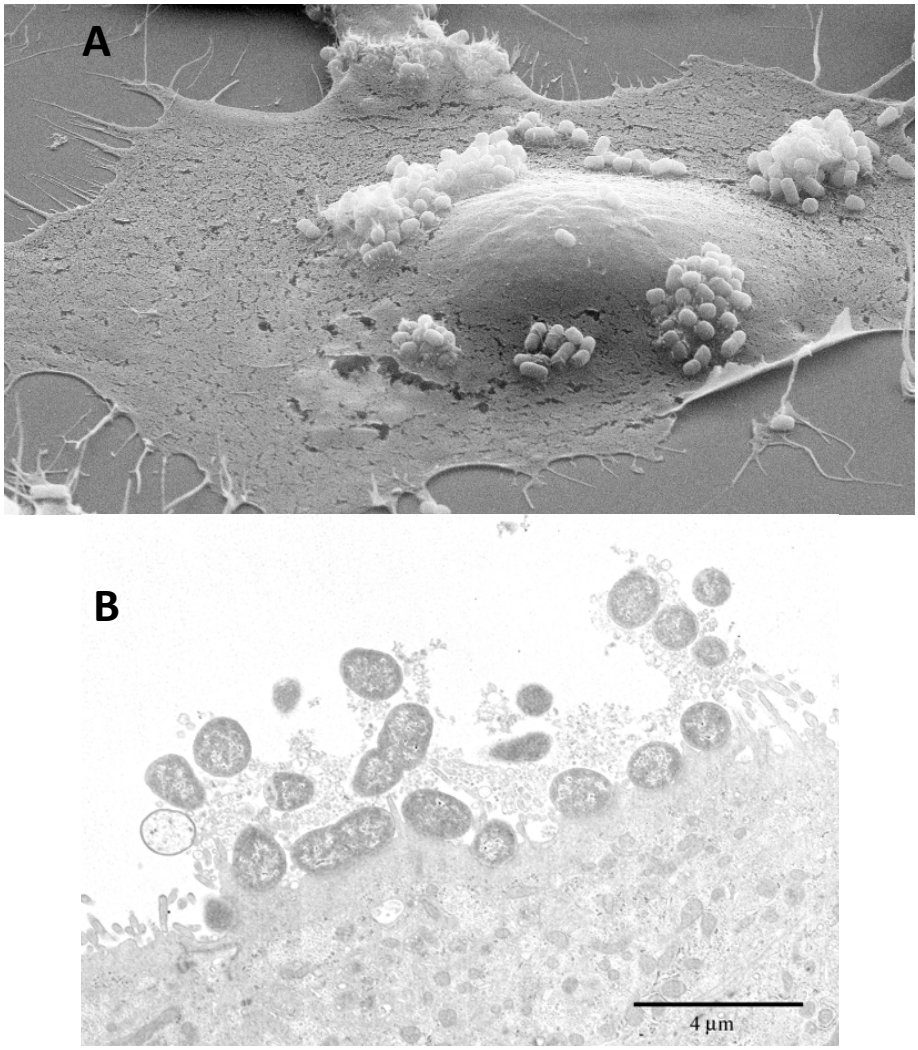


Fig. 2. Histopathological picture of EPEC infection on epithelial cells. SEM picture of EPEC E2348/69 microcolonies on the surface of an epithelial cell (**A**) and TEM of a Caco-2 cell layer infected with EPEC E2348/69 (**B**). Degeneration of microvilli and apical cytoskeleton is observed and pedestals are formed under bacteria attachment site (**B**). Micrographs were generated by L. Greune.

1.2.3. Adhesins of EPEC

As this study is mainly focused on the interactions of EPEC with host cells, further discussion (if not indicated otherwise) will be on this group. As for any successful pathogen

the first task for EPEC in the course of infection is to adhere to the targeted host cells. One of the proteins involved in adhesion is intimin. Intimin is encoded by the *eaeA* gene and is an afimbrial adhesin that mediates adhesion to epithelial cells. It mediates strong intimate adhesion of the bacteria to the host cells due to interactions with the Tir protein, located in the membranes of the epithelial cells. Tir is a bacterial protein which is translocated into the host cell cytosol and inserted into the cytoplasmic membrane with following Tir phosphorylation (see **1.2.4.2.**). Tir-intimin interaction occurs between the C-terminus of intimin and the middle region of Tir (Hartland *et al*., 1999; de Grado *et al*., 1999). Intimin was also shown to harbor a C-type lectin module in addition to two immunoglobulin-like domains (Kelly *et al*., 1999), which enables the protein to bind intestinal epithelial cells (IECs) in a lectin-like manner (Hartland *et al*., 1999). Gansheroff *et al*. showed that adhesion of EHEC to HEp-2 cells is decreasing in the presence of antibodies recognizing the C-terminus of intimin (Gansheroff *et al*., 1999). Now accumulating evidence supports the hypothesis that there is a putative receptor for intimin on the surface of epithelial cells. It was shown that purified intimin induces colonic hyperplasia in mice (Higgins *et al*., 1999; Torres *et al*., 2005) and activates T-cells (Goncalves *et al*., 2005; Torres *et al*., 2005). Additionally, a receptor was purified from HEp-2 cells which is recognized by EHEC intimin and which was later identified as nucleolin (Sinclair and O'Brien, 2002; Torres *et al*., 2005).

Microcolony formation of EPEC on target cell surfaces is greatly facilitated by the expression of a fimbrial adhesin named 'bundle-forming pilus (BFP)' that - besides mediating adhesion to host cells - is also 'bundling' the bacteria together (Cleary *et al.*, 2004). BFP are encoded on the characteristic virulence plasmid called 'EPEC adherence factor' plasmid (EAF) harbored by EPEC but not by ATEC. Besides the *bfp* operon required for pilus biosynthesis also the *per* operon is located on the EAF plasmid. Per is necessary for the regulation of bundle-forming pili biosynthesis and also for the activation of *eaeA* gene expression. Additional genes that encode potential virulence factors and various IS elements can also be found on the EAF plasmid (Tobe *et al.*, 1999).

In recent years, numerous outbreaks of diarrhoeal infections were associated with *E. coli* strains that are only rarely able to form microcolonies and mostly adhere as individual cells.

These strains have been coined 'diffusely adhering *E. coli* (DAEC)'. Some DAEC were later found to harbor the LEE pathogenicity island (see below) but to lack the EAF plasmid (Beinke *et al*., 1998). These strains are therefore not able to produce BFP and are usually referred to as atypical EPEC (aEPEC, ATEC). ATEC is now considered an emerging pathogen (Trabulsi *et al*., 2002; Nguyen *et al*., 2006). Daniel Müller *et al*. developed a fast method to differentiate EPEC, ATEC, and EHEC using multiplex PCR (MPCR) with four primers pairs – to *escV* (as a marker for LEE), *bfpB* (as a marker for EAF plasmid), *stx1* and *stx2* (for Shiga-toxins type 1 and 2). In this setup the *escV* marker has to be found for all LEE-positive strains, in EPEC strains there are additionally *bfpB*, and in EHEC there are additionally one or both of the *stx* genes (Müller *et al.,* 2006). Moreover, the MPCR was subsequently extended to allow for the differentiation of all known diarrhoeagenic pathotypes of *E. coli* in a one-step reaction (Müller *et al*., 2007).

1.2.4. The pathogenicity island (PAI): 'Locus of enterocyte effacement (LEE)'

1.2.4.1. Locus of enterocyte effacement and type III secretion system

In EPEC, all genes needed for the manifestation of the A/E phenotype are located on a 35-kb pathogenicity island (PAI) usually referred to as 'locus of enterocyte effacement' (LEE). The LEE is both necessary and sufficient for A/E lesion formation (Dean *et al*., 2005). It is located on the chromosome and as all PAIs has most likely been acquired by horizontal gene transfer from a so far unknown ancestor. Most of the genes on the LEE encode structural components, regulators, and substrates of the type III secretion system (TTSS). Expression of a TTSS enables Gram-negative bacteria to inject proteins directly into the cytosol of targeted host cells. TTSS are present in many Gram-negative bacterial pathogens, such as the human pathogens *Shigella spp*., *Salmonella spp*., *Pseudomonas aeruginosa*, and the plant pathogens *Erwinia spp*, *Pseudomonas syringae*, *Xanthomonas spp*., and many more (Hueck, 1998). Naturally, TTSS secreted effectors vary widely, but there are a few features common to all TTSS. As expected, structural proteins are largely conserved among TTSS harboring species.

Altogether the TTSS apparatus consists of approximately 20 proteins (Hueck, 1998; Winstanley and Hart, 2001; Gophna *et al*., 2003.). At the cytoplasmic site of the secretion machinery there is an ATPase that provides the energy needed for transport of the secreted proteins via the needle complex into the target cell. The ATPase is connected to the membrane-anchored proteins in the inner membrane. These proteins are reminiscent of the assembly apparatus for flagella, which suggests a common evolutionary origin. The inner membrane complex is connected to a multimeric protein in the outer membrane, which has homology to the PulD – secretin of the type II secretion systems (Hueck, 1998). In EPEC, a surface appendage of varying length is generated by the polymerization of the EspA protein (Knutton *et al*., 1998; Daniell *et al*., 2001; Cleary *et al*., 2004). The overall structure of the TTSS is reminiscent of a 'molecular syringe' as exemplified by the electron micrographs of purified TTSS apparatus of EPEC depicted on Fig. 3.

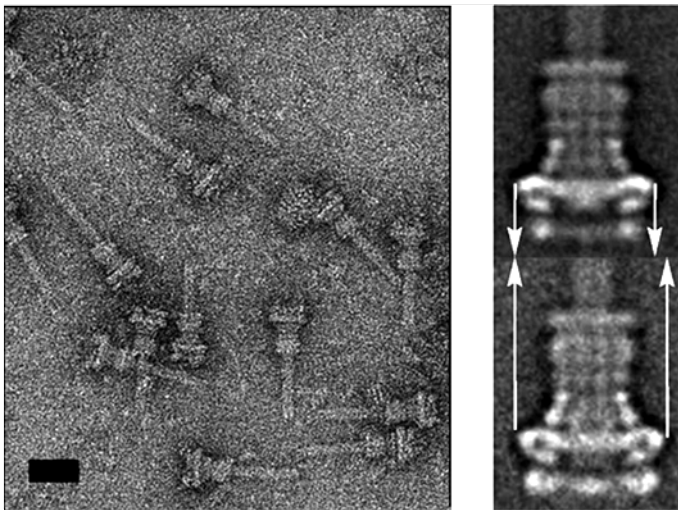


Fig. 3. Electron micrograph of purified TTS apparatus of *S. typhimurium*. The whole apparatus and membrane-anchored parts under higher magnification are shown. Taken from Marlovits *et al*., 2004.

One more TTSS feature distinguishing this secretion system from other, sec-dependent pathways is that it does not require canonical sequences on secreted proteins to be recognized as substrates for translocation. However, the N-terminus of the protein is usually

crucial for secretion, though TTSS appears to be relatively tolerant to amino acid changes in this region (Michiels and Cornelis, 1991). Some authors favored the hypothesis that the secretion signal might be located on the 5' end of the mRNA (Anderson and Schneewind, 1997). TTSS-dependent effector proteins of different species can exert many different functions as exemplified by the protein tyrosine-phosphatase YopH from *Yersinia*, the ADP ribosyltransferase ExoS from *P. aeruginosa*, or the integrin-binding protein IpaB of *S. flexneri* (Hueck, 1998). Fig. 4 demonstrates the organization of the LEE of EPEC E2348/69.

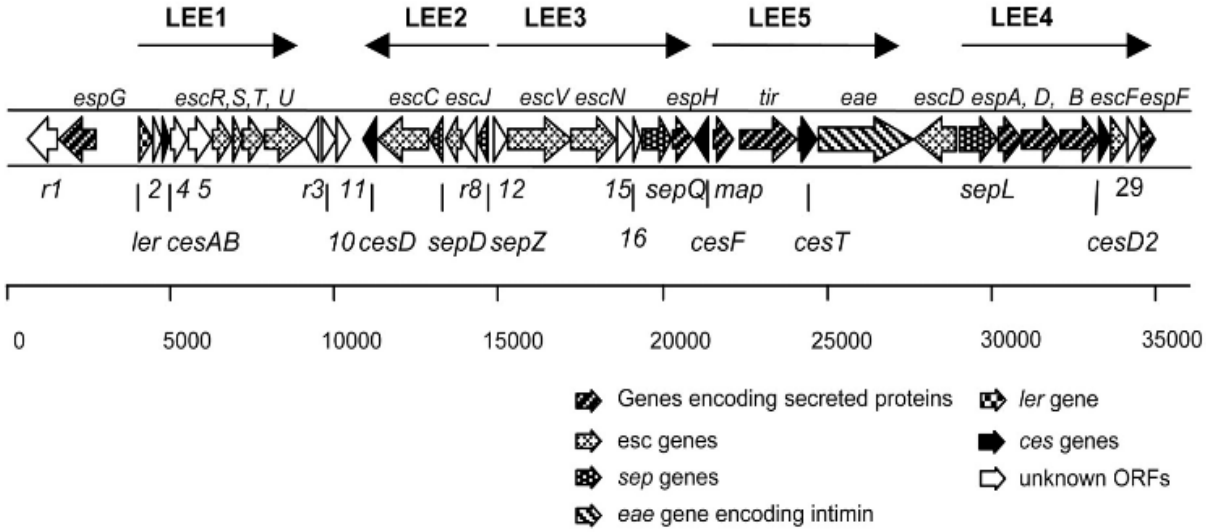


Fig. 4. LEE of the prototype EPEC strain E2348/69 (Gärtner and Schmidt, 2004).

Genes encoding proteins of associated functions such as structural, regulatory, chaperones and secreted proteins are arranged in operons in the LEE (LEE1-LEE5) (Winstanley and Hart, 2001). Genes designated *esc* (EPEC secretion) are encoding structural proteins of the TTSS which also have orthologs in the *Yersinia* TTS apparatus. *Sep* genes (secretion of EPEC proteins) have no orthologs in the *Yersinia* TTS apparatus, but were presumed to be part of the EPEC TTS apparatus as demonstrated later for SepQ, SepD (rorf6) and SepL (Deng *et al*., 2005; O'Connell *et al*., 2004). SepZ, however, was shown subsequently to be translocated into target host cells and most of the authors now refer to this protein as EspZ (Kanack *et al*.,

2005). *Esp* (EPEC secreted protein) genes are encoding effector proteins, though few of them have specific designations such as *tir* (translocated intimin receptor), or *map* (mitochondrial-associated protein). Gene of transcriptional regulator Ler, intimin-coding gene *eaeA* can also be found in the LEE, as well as chaperones genes designated *ces*. Ces AB (orf3) protein is a chaperone for EspA and EspB (Creasey *et al*., 2003b), CesT for Tir and Map (Elliott *et al*., 1999; Creasey *et al*., 2003a), CesD for EspD and EspB (Wainwright and Kaper, 1998), CesD2 for EspD (Neves *et al*., 2003), and CesF for EspF (Elliott *et al*., 2002).

1.2.4.2. TTSS effectors of EPEC

Currently known EPEC E2348/69 effectors are listed in Table 1.

EPEC effector	*Island location*	*Function*	*Subcellular target site (s)*	*Host partners*
Tir	LEE	Actin polymerization TJ disruption Cell detachment Microvilli effacement SGLT-1 inactivation PLC-γ phosphorilation Regulating effector activity Invasion non-polarized cells	Plasma membrane Cytoplasm	IQGAP-1 14-3-3tau Nck A-Actinin Talin Cortactin Vinculin Cytokeratin 18
Map	LEE	TJ disruption Filopodia formation Mitochondrial dysfunction Microvilli effacement SGLT-1 inactivation Invasion non-polarized cells	Mitochondria Actin Cytoplasm	EB50/NHERF1
EspB	LEE	Anti-phagocytosis Microvilli effacement Actin disruption Pore formation	Cytoplasm Plasma membrane	Antitrypsin Alpha-catenin Myosin

EspF	LEE	Apoptosis TJ disruption Microvilli effacement Microvilli elongation SGLT-1 inactivation Mitochondrial dysfunction Pedestal maturation Inhibition of NHE3 activity Membrane remodeling Aquaporin redistribution N-WASP activation	Mitochondria Cytoplasm TJ region Apical and lateral membrane	ABCF2 Actin ZO-1/ZO-2 Profilin Arp 2/3 Cytokeratin 18 N-WASP 14-3-3 Mito protein
EspH	LEE	Modulating actin dynamics Cytoskeleton disruption	Pedestals Plasma membrane	
EspZ (SepZ)	LEE	Unknown	Pedestals	
EspG	LEE	Microtubule disruption TJ disruption Paracellular permeability Aquaporin redistribution Stress fibers formation DRA transporter inhibition	Microtubule colonization	Tubulin
NleH1	PP2	Pro-inflammatory		
EspJ	PP2	Anti-phagocytosis		
NleB2	PP4	Unknown		
NleC	PP4	Unknown		
NleD	PP4	Unknown		
NleG	PP4	Unknown		
NleH2	PP6	Pro-inflammatory		
NleF	PP6	Unknown		
NleA (EspI)	PP6	Inhibition of protein secretion by interference with COPII	Golgi	Sec 24 PDZK 11 SNX 27 MAIS 3 TCOF 1
NleE2	IE2	PMN transepithelial migration	Nucleus	
EspG2/ Orf3	IE5	As with EspG		
NleB1	IE6	Unknown		
NleE1	IE6	PMN transepithelial migration	Nucleus	
EspL2	IE6	Unknown		
EspL1	IE2	Pseudogene		

NleB3	IE2	Pseudogene		
EspO	PP6	Pseudogene		
Cif	PP2	Pseudogene		
NleH3	PP4	Pseudogene		

Table 1. EPEC E2348/69 effectors (Dean and Kenny, 2009). Nle effectors are non-LEE encoded, PP loci are lambda-like prophage derived, and IE loci are integrative elements derived.

Scheme of EPEC effectors action on epithelial cell is represented on Fig. 5.

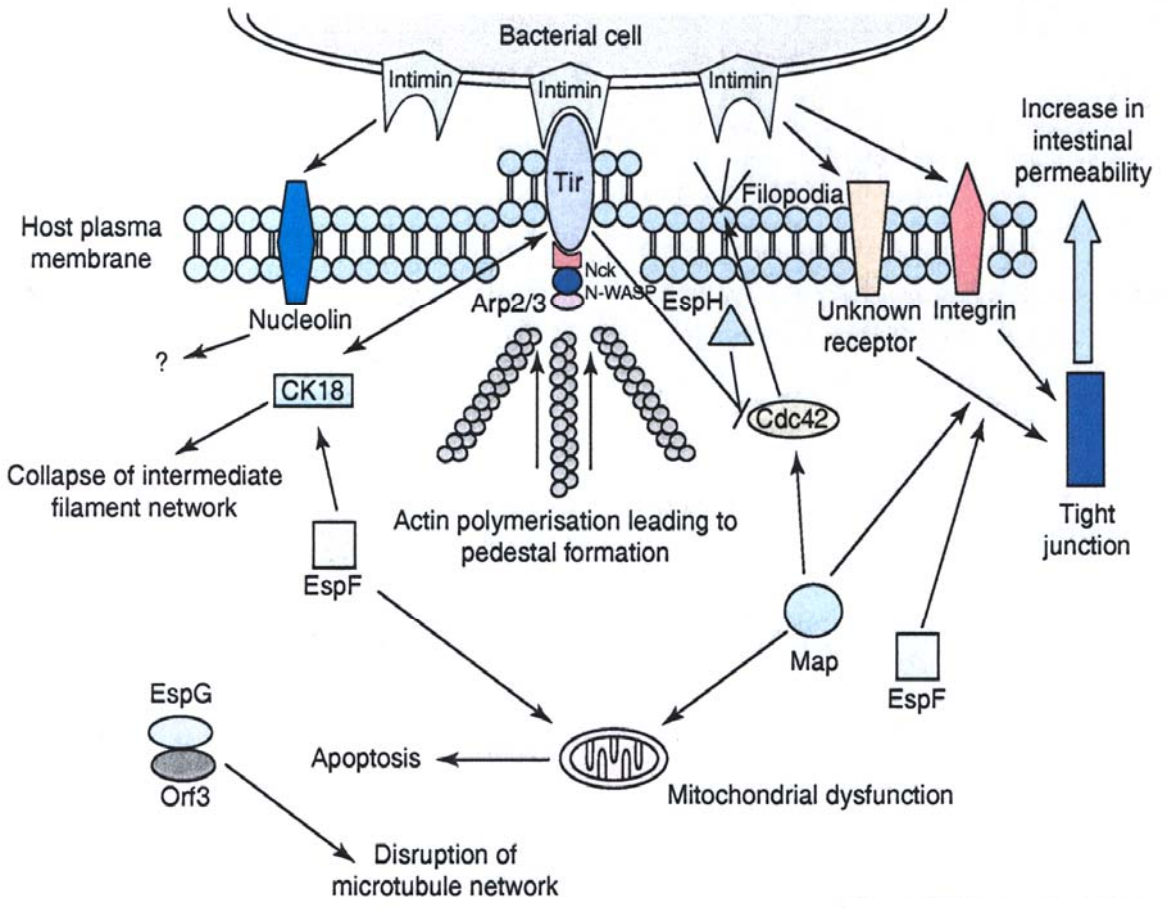


Fig. 5. Proposed scheme of EPEC TTSS-dependent effectors actions (Dean *et al*., 2005).

One of the first translocated proteins identified was the **Tir** where Tir stands for translocated intimin receptor. Tir was first discovered as the host cell receptor for intimin designated Hp90 and was thought to be a eukaryotic protein of the target cell. Only a few years later it became apparent that EPEC provide their own receptor as a bacterial protein that is injected into the host cell cytosol by the TTSS – hence translocated intimin receptor Tir. Tir-intimin interaction mediates intimate adhesion of bacteria to host cells, which is necessary for virulence. Following translocation into the host cell cytosol Tir is integrated into the eukaryotic membrane with its C-terminus located in the cytoplasm of the host cell where in EPEC it is phosphorylated by host tyrosine kinase(s) (Hartland *et al*., 1999). After phosphorylation Tir recruits a number of proteins to the bacterial attachment site, such as Nck, N-WASP, Arp 2/3 complex, and IQGAP-1 that are directly or indirectly involved in the induction of actin polymerization. Thus the orchestration of factors that are essential for actin polymerization in the end leads to actin polymerization underneath bacterial attachment sites and results in pedestal formation (Fig. 5). For Tir translocation the secretion of the proteins EspB, EspA and EspD is needed (Kenny *et al*., 1999). However, Michgehl *et al*. were able to show that Tir is also able to insert into the host cell membrane in an Esp-independent manner. Furthermore, these authors could show that the C-terminus of Tir is mediating its uptake and insertion, and such autointegrated Tir is functional as a receptor for intimin and also promotes pedestal formation. However, whether this additional pathway plays any significant role in vivo could not be resolved (Michgehl *et al*., 2006).

Map (mitochondrial-associated protein) is targeted to mitochondria causing their dysfunction, and is also involved in the formation of filopodia. Apparently, Map cooperates with EspF to cause these effects and in addition was also shown together with EspF and EspG to be involved in the breach of barrier functions.

EspB is a protein that can be considered as both being part of the apparatus and also acting as an effector. EspB is able to form pores with **EspD** in host cell membranes which is presumed to be necessary for translocation of other effectors (Ide *et al*., 2001), but it is also secreted and performs a set of functions in the cytoplasm (Table 1) (Dean and Kenny, 2009).

EspF is a 206 amino-acid protein with 3 proline-reach repeats that are supposedly involved in interactions with SH3-domain containing proteins. EspF was shown to be involved in cell death that has been described in EPEC infected cells without affecting adhesion (Crane *et al*., 2002). While cell death in cells infected with EPEC has features of both apoptosis and necrosis (Crane *et al*., 1999), expression of EspF in COS cells induces cell death with features of only apoptosis (Crane *et al*., 2002). EspF also recruits N-WASP and inhibits phagocytosis, latest in cooperation with EspB (Dean and Kenny, 2009).

A coordinated activity of EPEC effectors is observed with respect to actin dynamics in EPEC infections. During the first 20 min filopodia are formed which are resolved after translocation of several factors that subvert actin dynamics and lead to pedestal formation. One of the proteins involved in these processes is **EspH**. EspH is translocated into the host cell cytoplasm and localizes directly at the bacterial attachment site. EspH is involved in filopodia withdrawal and pedestal formation as EspH mutants are unable to resolve filopodia but still form pedestals. EspH over-expression leads to a strong repression of filopodia formation and, in addition, results in heightened pedestals (Tu *et al*., 2003). In general, it should be noted that EPEC effectors are often multifunctional, cooperative and also functionally redundant (Dean and Kenny, 2009).

Interesting results were obtained employing *in vitro* organ culture systems. Under these conditions it was shown that Tir mutants form so-called ’effacing bacterial footprints’ on the epithelial cells surface, where no pedestals are generated, but microvilli are effaced at the site of bacterial attachment. From these sites bacteria can easily detach as Tir-intimin interactions are not possible. EspB mutants, being necessary for TTS apparatus formation, induce the same phenotype as other TTSS deficient mutants, so-called ‘non-effacing footprints’. This phenotype is characterized by limited elongation of microvilli around attached bacteria that can easily detach as in this case adhesion only occurs through intimin. Map and EspH mutants produce so-called pedestal footprints, with the formation of typical A/E lesions. The only obvious difference from wild type strains is that on some of the pedestals bacteria were no longer present. Possible reasons for such an effect are not quite clear, as Tir and intimin expression and function should not be affected. It is obvious,

however, that Map and EspH are able to interfere somehow with bacterial adhesion, most probably by the modulation of Tir-intimin interactions. EspF mutants still form A/E lesions but are not able to elongate adjacent microvilli (Shaw *et al*., 2005).

A number of TTSS substrates have been identified that are encoded outside the LEE. Accordingly, these proteins were designated 'non-LEE encoded' or Nle. Later some of them were renamed Esp, such as EspJ and EspI, or were given a name reflecting their function, such as Cif. These factors are encoded on genetic loci associated with prophages (designated PP2, PP4 and PP6) or IS elements (designated IE2, IE5 and IE6) (Dean and Kenny, 2009; Table 1). Among the non-LEE encoded effectors is the cycle inhibiting factor **Cif**, which belongs to the cyclomodulin family of bacterial toxins and causes G1 and G2 cell cycle arrests. It was also shown to cause delayed apoptosis in the IEC6 rat intestinal epithelium cell line (in 48 hours) (Samba-Louaka *et al*., 2009). **NleE1** and **NleE2** effectors were reported to induce the transepithelial migration of PMNs (Zurawski *et al*., 2008). **EspI** (previously NleA) was proposed to be the key virulence factor in *Citrobacter rodentium* (Gruenheid *et al*., 2004). Other data suggest that it only contributes to the full virulence using the same model (Mundy *et al*., 2004). This effector is present in all diarrhoeagenic *E. coli* investigated, such as EHEC, EPEC, rabbit EPEC, and *C. rodentium* (Gruenheid *et al*., 2004). Kim *et al*. were able to show that EspI of EPEC inhibits protein secretion by binding to the Sec24 protein that is involved in intracellular vesicular transport of proteins (Kim *et al*., 2007).

Three to four major steps can be described in the EPEC infection process (Nataro and Kaper, 1998). The first step involves the adherence of bacteria to targeted host cells, initially mediated through BFP and intimin. In the second step, the TTSS apparatus is assembled and secretion of effectors begins. After the Tir-intimin connection is established intimate adhesion occurs. During this process also various effectors are secreted including Map, which triggers filopodia formation by activating Cdc42. Map is also interacting with ezrin and activates the RhoA/ROCK cascade which stabilizes filopodia. This stage ends when Tir is phosphorylated and recruits Nck, as this will lead to filopodia withdrawal and pedestal formation (Berger *et al*., 2009) in parallel to all other effects caused by EPEC infection which

is regarded as the third and the last stage of infection. Filopodia withdrawal cannot occur if Tir is not phosphorylated and/or Nck proteins are not present (Berger *et al*., 2009).

Additional activities reported for EPEC include the induction of cell death (Abul-Milh *et al*., 2001) in HeLa cells, antiphagocytosis, the prevention of opsonophagocytosis in immune cells (Goosney *et al*., 1999; Celli *et al*., 2001; Marches *et al*., 2008), and the hemolysis of erythrocytes (Warawa *et al*., 1999; Ide *et al*., 2001). Cell death of HEp-2, HeLa, and Caco-2 epithelial cell lines was shown to be dependent on BFP and to some extent on intimin expression. Strains lacking BFP showed significantly less cell death then wild type strains. Both apoptosis and necrosis were shown to take place in infected cells, in approximately the same ratios (around 15% for each of the cell types). Interestingly, BFP mutants cause significantly less apoptotic events and only weakly influenced necrotic cell death, whereas in infections with intimin mutants only the necrotic cells death events were significantly reduced (Abul-Milh *et al*., 2001).

EspF and the non-LEE encoded effector EspJ are involved in the antiphagocytotic effects. EspF prevents the actual phagocytosis of the bacteria, therefore bacteria are attached but not internalized and are therefore still able to promote the formation of A/E lesions and to mediate additional effects on the host cells. Antiphagocytotic effects were shown to involve PI-3-kinase dependent pathways, which EPEC is able to inhibit (Celli *et al*., 2001). EspJ prevents opsonophagocytosis of red blood cells by macrophages through both Fcγ3 and CR3 receptors (Marches *et al*., 2008).

Hemolytic activity of EPEC was shown to require bacteria-cell contact and de novo protein synthesis. This activity is also TTSS-dependent and mutants of EspA, EspB, and EspD but not of any other effector abolish this effect. EspB and EspD are predicted to have pore-forming activity which might be instrumental for this effect. EspA is possibly needed just for the delivery of proteins to the host cell membranes. As no other proteins except EspA, EspB, and EspD were found, it could be that these proteins are either directly involved in lysis or that there are some other not yet identified effector proteins responsible for this effect (Warawa *et al*., 1999; Ide *et al*., 2001).

1.3. The gut normal microflora as an integral part of the gastrointestinal barrier

1.3.1. Role of gut microbiota in defense to enteric infections

Most of the studies on EPEC pathogenesis were conducted using epithelial cell lines *in vitro*. However, *in vivo* the situation is certainly more complex. The very first barrier that enteropathogens face in the gut is the normal microbiota. Newly incoming pathogens must compete with earlier arrivals and commensals for available space, niches, and resources. There are around 400 species in the normal human gut, which are very well adapted to this habitat based on long-term co-evolution. In order to protect their respective niches commensals usually express factors supporting their survival which includes factors involved in iron uptake, adhesion to and modulation of epithelial layer functions (Hooper and Gordon, 2001; Sonnenburg *et al.*, 2005; Zoetendal *et al.*, 2008). Therefore, commensals are maintaining a delicate balance with the host's defense systems and are contributing to the education and homeostasis of the low-level 'inflammatory conditions' of the gut-associated lymphoid tissues (GALT). Due to these properties some commensals have actually found therapeutic and/or prophylactic applications and – in some cases – have been used for decades as 'probiotics'. Probiotics are defined as microorganisms that when applied in adequate numbers exert a health benefit to the host. The longest known probiotics are Gram-positive bacteria such as *Lactobacilli* and *Bifidobacteria*. The best known probiotics among Gram-negative bacteria are *E. coli* species, such as *E. coli* M17 and *E. coli* Nissle 1917 (EcN) which is the active compound of the Mutaflor® drug that is available in Central Europe 'over-the-counter'.

1.3.2. *E. coli* Nissle 1917 (EcN) as a probiotic *E. coli* strain

The EcN strain is reported to exert a various positive effects on the intestinal barrier and, moreover, has been shown to reduce also inflammation in the gut (Grabig *et al.*, 2006). Further effects include the improvement of epithelial barrier integrity (Zyrek *et al.*, 2007), and the restriction of invasion and proliferation of pathogenic bacteria (Altenhoefer *et al.*, 2007; Reissbrodt *et al.*, 2009; Leatham *et al.*, 2009). In a mouse sepsis model (Arribas *et al.*, 2009) the EcN strain induced ß-defensin-2 secretion (Wehkamp *et al.*, 2004). Induction of β-defensin-2 was reported for quite a few probiotic bacteria including EcN but not for pathogens or non-pathogenic commensal strains. EcN is inducing β-defensin-2 expression and secretion in Caco-2 cells. The induction was shown to be dependent on NF-κB and AP-1 as specific inhibitors of NF-κB (Helenalin) and c-Jun N-terminal kinase abolished the effect (Wehkamp *et al.*, 2004). Later flagellin was found to be responsible for the effect (Möndel *et al.*, 2009). Using INT407 cells (human embryonic intestinal epithelial cells) EcN was found to protect against invasion of several pathogens such as *S. enterica sv typhimurium*, *Y. enterocolitica*, *S. flexneri*, *L. pneumophila*, and *L. monocytogenes*. 45 – 65% reduction in invasion efficiency was observed, and this effect was not due to direct bactericidal effects of EcN, as the viability of pathogenic strains was not altered (Altenhoefer *et al.*, 2003). EcN was also shown to inhibit TNF-α-induced IL-8 transactivation in epithelial cells (HCT15, human colonic epithelium cell line). For this live EcN bacteria are needed, but interestingly contact to epithelial cells is not necessary. Inhibition is connected to IL-8 promoter suppression and is not caused by blocking of NF-κB (Kamada *et al.*, 2008). Among the fitness factors that strain expresses are iron scavengers and microcins negatively regulated by iron presence (Valdebenito *et al.*, 2006; Patzer *et al.*, 2003).

1.3.3. Microcins as *E. coli* Nissle 1917 'fitness factors'

Microcins are a group of low-molecular weight (generally less than 5000) antibacterial peptides that are secreted by some members of the *Enterobacteriaceae* family. The

antibacterial activity of microcins is usually restricted to species of the same family. The structure and mechanism of action of the different microcins are quite diverse which distinguishes them from bacteriocins secreted by Gram-positive bacteria (Drider *et al.*, 2006; Gillor *et al.*, 2008). Among the best studied microcins are J25, acting on the RNA polymerase (Delgado *et al.*, 1999; Yuzhenkova *et al.*, 2002), E492, acting on the inner membrane by a TonB-dependent mechanism (Pugsley *et al.*, 1986; Pons *et al.*, 2002; Destomieux-Garzon *et al.*, 2003), and C51, inhibiting translation by blocking aspartyl-tRNA synthetase (Kurepina *et al.*, 1993; Metlitskaya *et al.*, 1995; Fomenko *et al.*, 2001). EcN was reported to have bactericidal activity (Nissle, 1925; Papavassiliou, 1959, 1961) that was later connected to the production of two microcins, M and H47 (Patzer *et al.*, 2003). Genes encoding both microcins and the associated immunity proteins are located together on the chromosome of the microcin-producing strain. Scheme of their location is shown in Fig. 6.

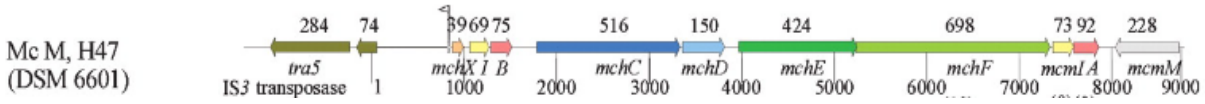


Fig. 6. Genetic locus of EcN 1917 containing genes coding microcins and their immunity proteins. Taken from Patzer *et al*., 2003.

Further investigation of these microcins has been severely hampered by their remarkable instability. Recently they were purified. Presence in susceptible strains of at least one siderophore receptor (FepA, Fiu or Cir) and inner membrane protein TonB were shown to be necessary for microcins H47 and M action (Patzer *et* al., 2003; Vassiliadis *et al*., 2009).

Microcin H47 was shown to need the H^+ pump activity of ATPase, though it is currently unknown whether ATPase is its final target (Trujillo *et al.*, 2001; Rodriguez and Lavina, 2003). An immunity protein for the H47 microcin is encoded by the *mchI* gene (Fig. 6.). MchI is a 69-amino acid protein with two predicted transmembrane domains pointing to a membrane location for this protein although the mechanism of MchI protective action remains unclear. It has similarities to proteins that are involved in the respiratory chain, such as cytochrome b from red algae. However, there is not much known about the activity of the H47 microcin

itself although an activity towards biological membranes has been proposed (Rodriguez and Lavina, 1998). Up to now, the mechanism of microcin M action has remained elusive.

1.4. Host-pathogen interactions at the gastrointestinal barrier

1.4.1. Role of epithelium in antibacterial defense

In addition to the interactions with the resident microflora incoming pathogens interact with the epithelial barrier where they have to overcome several defense mechanisms of the host. A scheme of the organization of the intestinal barrier with its major components is depicted in Fig. 7.

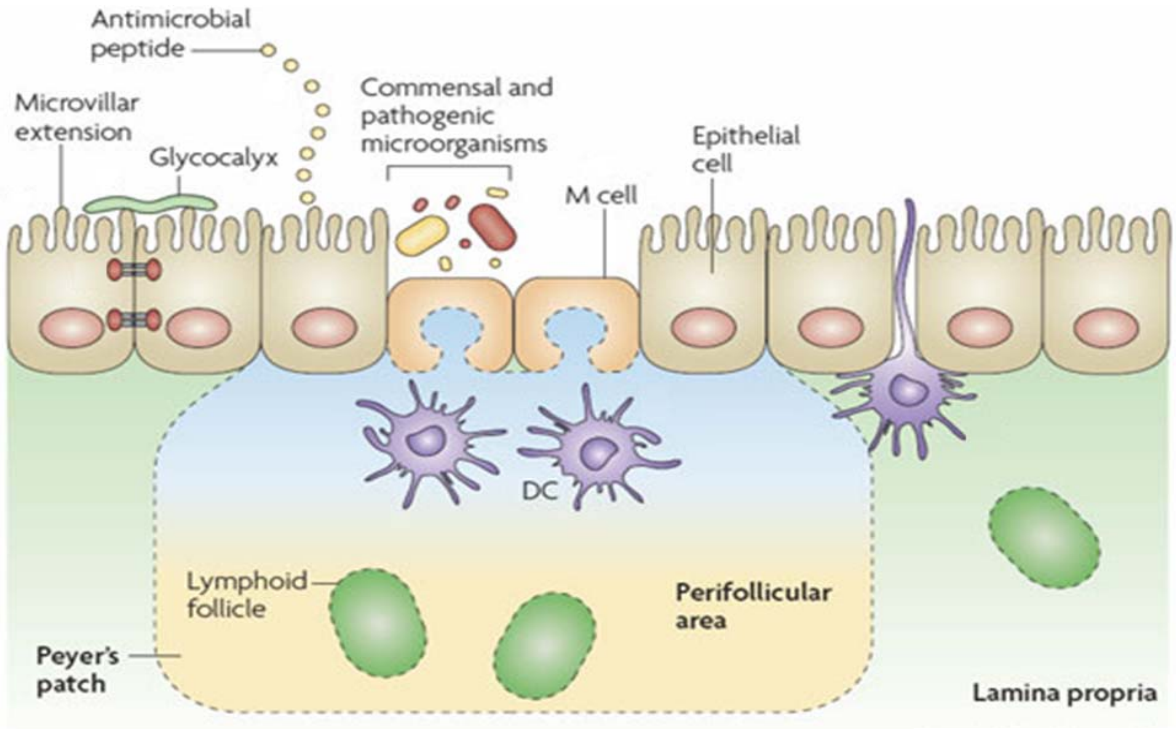


Fig. 7. Main components of the gastrointestinal barrier involved in gut immunity (Artis, 2008).

Enforcements of the intestinal barrier also include physical and chemical barriers such as the mucus layer formed by glycoproteins called mucins. Some mucins are expressed constitutively, others changes the expression upon an infection (Li *et al*., 1998; Linden *et al*., 2008). There are several mucins produced by intestinal epithelial cells, some of them stay bound to epithelial cells, others are secreted and form a viscous gel all over the apical surface of epithelial cell linings (Atuma *et al.*, 2001; Hattrup and Gendler, 2008). Penetration of the mucus layer is facilitated by bacterial mucinases or other lytic enzymes that are able to cleave and destroy mucins. Commensal bacteria that are unable to get through the mucus layer are constantly washed away by peristalsis, a process that hampers their multiplication and keeps their numbers below a potentially dangerous threshold (Vance *et al.*, 2009).

Innate immunity mechanisms of protection include the production of defensins (Menendez and Finlay, 2007) and the secretion of immunoglobulin A (IgA). For IgA production antigens have to be sampled at the luminal side of the intestinal barrier (Mora et al., 2006). Acquisition of bacteria from the luminal side can proceed by sampling of the luminal content by M cells (membranous or microfold cells). M cells are characterized by stumpy and shortened microvilli and are interspersed in the follicle-associated epithelium (FAE) of Peyer's patches and also in the epithelium of neighbouring villi (Owen and Jones, 1974; Nicoletti, 2000; Janga *et al.*, 2004). M cells are able to transcytose phagocytosed particles or bacteria from the gut lumen directly to antigen-presenting cells (macrophages, dendritic cells) harboured in a basolateral invagination or pocket (Miller *et al.*, 2007; Kraehenbuhl and Neutra, 2000).

1.4.2. Role of dendritic cells at the gastrointestinal barrier

Another cell type able to sample luminal contents are dendritic cells which form protrusions from the basolateral side of the epithelial monolayer to the luminal side – apparently without disturbing the tight junctions (TJs) (Steinman, 1991; Rescigno *et al.*, 2001). This is achieved via the expression of TJs molecules by DC which were shown to produce claudin-1, E-cadherin, ß-catenin, occludin, and ZO-1 at different time-points after

addition to intestinal epithelial cells (IECs) layers. ZO-1 protein expression was only observed after bacteria were added to the apical side of the epithelial cell layer (Rescigno *et al.*, 2001). DC take up bacteria and migrate to the lymph node for antigen presentation. DC are the most abundant immune cells at mucosal surfaces as well as in the skin representing the first frontiers against infection. They are recruited in large numbers upon contact with potentially harmful organisms (Chieppa *et al.*, 2006; Niess *et al.*, 2005). Commensal bacteria were shown to survive in DC of mice for up to 60 hours. They are then transported for antigen presentation to mesenteric lymph nodes. However, they do not proceed further, therefore inducing only local responses (Macpherson and Uhr, 2004 a, b; Sansonetti, 2001).

Before contact with antigens, DC are immature, i.e. they have high capability for phagocytosis and antigen degradation, do not express costimulatory molecules, and exhibit low migratory properties. After contact with antigens, DC maturate, indicated by the expression of several costimulatory molecules such as CD83, CD86. In addition, their migratory potential is improving whereas phagocytosis and antigen degradation efficiency decrease. Which maturation markers DC will express and to what extent can vary widely depending on the nature of the antigen and on the signals received from the neighbourhood. This is also important for the selection of certain population of T cells.

It was shown, for example, that under normal conditions (only normal microflora present at the luminal side and no danger signals sensed by IECs) IECs are producing certain interleukins which are 'instructing' DC in a way that they are only able to induce Th2, Treg responses or activate B cells for IgA production (Iliev *et al.*, 2007). The complex role of signals derived from epithelial cells needed for the generation of proper immune responses can be viewed in Fig. 8.

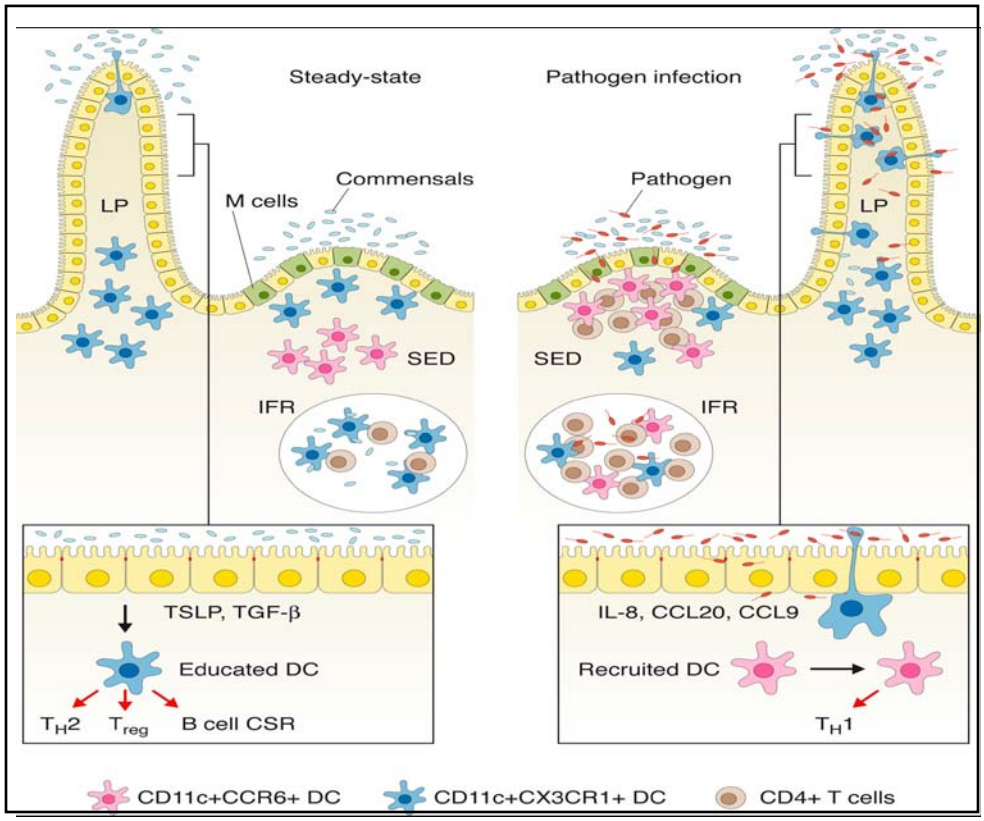


Fig. 8. Differences in the ability of DC to prime certain types of lymphocytes depending on cytokines they are exposed to (Iliev *et al.*, 2007). SED – subepithelial dome; IFR – interfollicular T cell areas; CSR – class switch recombination.

As an example of such 'conditioning' cytokines thymic stromal lymphopoietin (TSLP) can be reviewed in more details, as numerous groups have shown its utmost role in instructing DC. Stable levels of TSLP are needed to avoid unnecessary inflammatory responses. Low levels of TSLP are associated with Crohn's disease (Rimoldi *et al.*, 2005) whereas elevated levels are often observed in patients with pathologic conditions such as atopic dermatitis (Soumelis *et al.*, 2002; Yoo *et al.*, 2005) and allergic asthma (Al-Shami *et al.*, 2005; Zhou *et al.*, 2005). Once conditioned in this way, DC lose the ability to induce Th1 inflammatory responses even when being exposed to proinflammatory stimuli such as LPS (Iwasaki *et al.*, 1999). TSLP is expressed mostly by epithelial cells, where the highest expression can be observed in the lung, skin, and gut (Reche *et al.*, 2001). TSLP was first identified in the thymus, although its

function there still remained unknown. It can only be hypothesized that TSLP in the thymus medulla activates DC that positively select self-reactive Treg cells to maintain self-tolerance (Watanabe *et al.*, 2005). TSLP-activated DC induce a very strong expansion of CD4+ T cells which have a memory cell phenotype and can further be differentiated in Th1 or Th2 cells. As can be seen in Fig. 8, TSLP-conditioned DC favor Th2 cell lineage for priming, main ligand responsible for such selection was shown to be OX40 (Ito *et al.*, 2005). The TSLP receptor is a heterodimer that includes the specific TSLP-R subunit and IL-7-Rα subunit. As well as IL-7, TSLP induces STAT-5 signalling, however, without activating JAK kinase, as it was reported for IL-7 (Liu *et al.*, 2007).

In case of infection, IECs start to secrete numerous factors that attract immune cells including DC. The newly incoming, non-conditioned DC are able to induce Th1 responses (Sansonetti, 2004; Iliev *et al.*, 2007). Infection is sensed by IECs by so-called pattern-recognition receptors (PRRs) including toll-like receptors (TLRs), most of which are located at the basolateral surface or in the cytoplasm to avoid initiating active immune responses to the gut microflora but being able to react immediately to invasive pathogens (Rakoff-Nahoum *et al.*, 2004).

One of the best-studied responses is the secretion of IL-8, which is induced, for example, in response to flagellin triggering TLR5. IL-8 is a chemoattractant for leukocytes and causes neutrophil infiltration with further acute inflammation and tissue damage. Anti-IL-8 antibodies are able to suppress inflammation in a various conditions connected with acute inflammation (lung reperfusion injury, LPS-induced dermatitis, etc.) in murine models (Harada *et al.*, 1994). The biological activity of IL-8 is mediated through the G-protein-coupled receptors CXCR1 and CXCR2, which upon activation signal downstream to activate such factors as NF-κB, AP-1, STAT-3, and β-catenin (Waugh and Wilson, 2008).

In summary it is quite apparent that the intestinal barrier is a complex frontier where a well-balanced structure is maintaining gut homeostasis by promoting tolerance to microflora and - at the same time - by being able to induce rapid immune responses to pathogenic microorganisms. These functions are only possible due to an intricate interplay between

various cell types, including intestinal epithelial cells, leukocytes and lymphocytes, and also bacterial cells comprising normal gut microflora.

1.5. Aim of this study

The goal of this study was the characterization of molecular and cellular interactions of immune cells at the intestinal barrier with enteric *Escherichia coli* using a cell culture or tissue culture model system.

For this special attention should be directed to the interactions of primary human monocyte-derived DC and, alternatively, of the monocytic cell line THP-1 as model immune cells. On the microbial side interactions with several *E. coli* strains including the prototype EPEC strain E2348/69 (serotype O127:H6) as a representative pathogen, the probiotic *E. coli* Nissle 1917 (serotype O6:K5:H1), and the common laboratory strain C600 for comparison were investigated. In order to mimic the interaction of DC with intestinal epithelial cells, a co-culture model integrating polarized Caco-2 cells and freshly isolated human monocytes was established using a Transwell filter system. The response of such a co-culture model to infections with different *E. coli* strains was analyzed using immunofluorescence and electron microscopy techniques. Major cellular effects investigated included pedestal formation and cell death. In addition, to obtain a more global view on changes in gene expression in response to pathogenic or commensal bacteria microarray technology and qRT-PCR were applied. Antagonistic effects of EcN that is known to produce microcins towards different diarrhoeagenic *E. coli* were assessed.

2. Materials and methods

2.1. Materials

2.1.1. Chemicals and materials

Chemicals and materials used in this study are listed in Table 2.

Name	*Manufacturer*
Poly-L-lysine 0.01%, ready solution	Sigma
PFA powder	Merck
BSA H1 powder	Gerbu
Glycin powder	Aldrich
$CaCl_2$*$2H_2O$ salt	Merck
Tri reagent ready solution	Sigma
Percoll ready solution	GE Healthcare
Gentamycin sulfate salt	Sigma
Kanamycin A disulfate salt	Gerbu
Nalidixic acid powder	Merck
Mounting medium, ready solution	Dako Cytomation
Glass slides, ca. 76x26 mm	R. Langenbrinck
Glass coverslips, D=12 mm	Neubauer
Glass counting chamber, depth 0.0025 mm^2	Neubauer

Table 2. Chemicals and materials.

2.1.2. Buffers, medium, solutions

ddH_2O was used for preparation of all solutions, these were then autoclaved or filtered under sterile conditions.

Standard I-medium for cultivation of bacteria.

25 g per liter of standard I nutrient broth (Merck).

Agar plates

15 g agar (AppliChem) per liter of standard I-medium. When necessary, sterile antibiotic solution was added to the agar after autoclaving. Concentrations of antibiotics used: kanamycin – 30 µg/ml; nalidixic acid – 10 µg/ml.

D-PBS 10x

Per 1 liter

$Na_2HPO_4xH_2O$	14.24 g
KH_2PO_4	2 g
NaCl	81.9 g
KCl	2 g

When necessary the 10x solution was diluted to 1x, autoclaved and pH was checked (required pH is 6.8).

Binding buffer for staining with anti-annexin V antibodies 10x

Water solution containing

HEPES	0.1 M
NaCl	1.4 M
$CaCl_2$	25 mM

4% PFA

10% PFA was prepared in 3/4 of the final volume of PBS and dissolved at 60°C with constant stirring. 1M NaOH was then added until the solution become transparent. PFA was then aliquoted and frozen at -20°C. When needed the solution was defrosted and PBS was added until the 4% solution was obtained.

0.2% Glycin.

10% glycin in PBS was prepared, aliquoted and frozen at -20°C. When needed the solution was defrosted and PBS was added until the 0.2% solution was obtained.

1% and 0.1% BSA

10% BSA in PBS was prepared, centrifuged, aliquoted and frozen at -20°C. When needed the solution was defrosted and PBS was added until the 1% or 0.1% solution was obtained.

1M NaOH

1M NaOH in ddH_2O.

Gentamycin solution

10 mg/ml gentamycin sulfate in ddH_2O.

Kanymycin solution

10 mg/ml kanamycin sulfate in ddH_2O.

Nalidixic acid solution

10 mg/ml nalidixic acid in ddH_2O.

2.1.3. Commercially available kits

Kits used in this work are listed in Table 3.

Name	*Manufacturer*
Transcriptor high fidelity first strand cDNA synthesis kit	Roche
LightCycler fast start DNA Master^{PLUS} SYBR green I	Roche
High purity RNA isolation kit	Roche
Cytotox96 non-radioactive Cytotoxicity assay kit	Sigma

Table 3. Kits.

2.1.4. Antibodies

Antibodies and fluorescent reagents used in that work are listed in Table 4.

Name of antibody/reagent	*Conjugate*	*Manufacturer*	*Application*
Primary antibodies and fluorescent substances			
Rabbit antihuman CD14	PE	Acris	FC
Mouse antihuman CD11c	-	Myltenyi Biotech	FC
Mouse antihuman annexinV	FITC	AbD Serotec	FC
Mouse antihuman CD86	FITC	Invitrogen	FC
Propidium Iodide (PI)	-	Sigma	FC
DAPI	-	Pierce	IF
Carboxyfluorescein succinimydil esther (CFSE)	-	Sigma	IF
Phalloidin	Texas Red	Molecular Probes	IF
Mouse anti-EPEC Tir	-	Raised in mouse	IF
Secondary antibodies			
Goat anti-mouse antibodies	Cy2; Cy3	Dianova/Jack	IF
Donkey anti-rabbit antibodies	Cy3	Dianova/Jack	IF
Streptavidin	AlexaFluor647	Molecular Probes	IF

Table 4. Antibodies. FC – flow cytometry, IF – immunofluorescence.

2.1.5. Bacterial strains, cell lines and plasmids

Bacterial strains and plasmids used in this study are represented in Table 5.

strain or plasmid	*relevant characteristics*	*source or reference*
strains		
E. coli Nissle 1917	probiotic, microcins H47 and M secretion; O6:K5:H1	Mutaflor/Ardeypharm, Germany
SK22D	EcN Δ*mchDEF*	Courtesy of U.Dobrindt; Patzer *et al*., 2003

E. coli K12 C600	F- *tonA21 thi-1 thr-1 leuB6 lacY1 glnV44 rfbC1 fhuA1 lambda-*	ATCC, US Pat. 4.710.473
E. coli B BL 21	*F– ompT gal dcm lon hsdSB(rB-mB-) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5*	Studier and Moffat, 1986; Grodberg *et al*., 1988
EPEC E2348/69	O127:H6 serotype wild type diarrhoeagenic strain	Gift from Rowe, London Knutton *et al*., 1987 a, b
cfm 14-2-1	EPEC E2348/69 *escN*::TnphoA	McDaniel *et al*., 1995; Courtesy of M.Donnenberg;
cfm 30-5-1	EPEC E2348/69 *sepZ*::TnphoA	McDaniel *et al*., 1995; Rabinowitz *et al*., 1996; Courtesy of M.Donnenberg;
10-5-1	EPEC E2348/69 *eaeA*::TnphoA	Donnenberg *et al*., 1990; Courtesy of M.Donnenberg;
UMD870	EPEC E2348/69 *espD::aphA-3*	Lai *et al*., 1997
SE896	EPEC E2348/69 *Δtir*	Elliott *et al*., 1999; Courtesy of J.Kaper;
UMD864	EPEC E2348/69 *ΔespB*	Donnenberg *et al*., 1993; Courtesy of Br. Kenny
Map mutant	EPEC E2348/69 *Δmap*	Kenny and Jepson, 2000; Courtesy of Br. Kenny
EspF mutant	EPEC E2348/69 Δ*espF*	Warawa *et al*., 1999; Courtesy of Br. Kenny
EPEC Cigleris	O128:H2	Courtesy of Fr. Orskov, Copenhagen
EPEC 5274	O125	Courtesy of H. Karch, Münster
ATEC 9812	O128:H2	Institute of Hygiene, Heidelberg
ATEC 3431-4/86	O8:H-	Courtesy of L. Trabulsi, São Paulo
ATEC B6	O26:K60	Institute of Medical Microbiology, Münster
ATEC 10459	O55:H-	Institute of Hygiene, Heidelberg
ATEC 0181-6/86	O119:H9:K61	Courtesy of L. Trabulsi, São Paulo
ATEC 0036/78	O55:H6	K. Wachsmuth, Dept. Health and Human Services, Centers for Disease Control, Atlanta GA 30333
ATEC 660-79	O55:H7	K. Wachsmuth, Dept. Health and Human Services, Centers for Disease Control, Atlanta GA 30333
ATEC 15740	O55:H7	Institute of Hygiene, Heidelberg
ATEC 15716	O55:H7	Institute of Hygiene, Heidelberg
ATEC 1972-1/85	O127:H40	Courtesy of L. Trabulsi, São Paulo
ATEC 11638	O128:H8	Institute of Hygiene, Heidelberg
ATEC 10419	O128:H2	Institute of Hygiene, Heidelberg
EAEC 260	O86	Courtesy of Helge Karch, Münster

EAEC 02/1850	human isolate	Courtesy of T. Gomes, São Paulo
EAEC Fu 297-1	human isolate	Courtesy of T. Gomes, São Paulo
EAEC Fu 214-4	human isolate	Courtesy of T. Gomes, São Paulo
EAEC Fu 154-2	human isolate	Courtesy of T. Gomes, São Paulo
EIEC 02-10479	O124:H-	RKI, Wernigerode
EIEC 99-10280	O143:H-	RKI, Wernigerode
EIEC 99-10281	O144:H-	RKI, Wernigerode
EIEC 99-10282	O152:H-	RKI, Wernigerode
EIEC 99-10283	O164:H-	RKI, Wernigerode
EIEC 00-4745	O164:H-	RKI, Wernigerode
EHEC 2996/96	O177:H-	Courtesy of H.Karch
EHEC EDL933	O157:H7	Courtesy of H.Karch
EHEC 493/88	O157:H-	Courtesy of H.Karch
EHEC 5720/96	O26:H11	Courtesy of H.Karch
Cell lines		
THP-1	Monocytic leukemia, human	ATCC TIB-202
Caco-2	Colon carcinoma, human	DSMZ Braunschweig
HBMEC	Human brain microvascular endothelial cells	Rood *et al*., 2000
plasmid		
pVLT33-GFP	Resistance to kanamycin, GFP expression	

Table 5. Bacterial strains and plasmid.

2.1.6. Cell culture

Reagents, additives and materials used in the cell culturing are listed in Table 6.

Name	*Manufacturer*
RPMI 1640	PAA
DMEM	PAA
MEM Spinner	Sigma
HEPES 10x	PAA
Non-essential amino acids (NEAA) 10x	PAA
Vitamins 10x	PAA
FBS	PAA
Nu-Serum	BD
Transferrin	Sigma
GM-CSF	Peprotech
IL-4	Peprotech

Penicillin/Streptomycin mixture 10x	PAA
Lymphocyte separation medium	MP Biomedicals
Extracellular matrix proteins mixture	Invitrogen
3 µm pore size filters for 24-well plate	Costar
12-well and 24-well plates, cell culture treated	Costar
Cell culture flasks, 75 cm^2	Greiner BioOne

Table 6. Medium and supplements used in cell culture.

2.2. Methods

2.2.1. Maintaining cell lines

The THP-1 cell line was maintained in RPMI 1640 medium supplemented with 10% FCS and 100 µg/ml penicillin and streptomycin. Per 75 cm^2 flask 3 ml of medium were added every second day. Once a week the cells were split as follows: 1 ml of cell suspension (out of 19) were centrifuged at 400xg for 4 min and re-suspended in 10 ml of fresh medium.

Caco-2 cells were grown in DMEM High Glucose medium supplemented with 10% FCS, 100 µg/ml penicillin and streptomycin, non-essential amino acids (NEAA) and human transferrin. When cells reached 80 – 90% confluency they were split as follows: after removal of the medium, the cells were washed once with PBS and gently shaken for 2-3 min. Then 3 ml (per 75 cm^2 flask) 0.025 % trypsin were added and the cells were incubated for 2-3 minutes at RT and then 2-5 minutes at 37°C. Afterwards the cells were detached from the flask surface by washing with 3-10 ml of fresh medium (if necessary after shaking). 1 ml of the suspension (or another volume if a different dilution was desired) was then centrifuged for 4 min at 400xg at RT, and the pellet was resuspended in 10 ml of fresh medium.

Human brain microvascular endothelium cells (HBMEC) were grown in RPMI 1640 medium supplemented with 10% FCS, 10% Nu-serum, vitamins, 100 µg/ml penicillin and streptomycin, and NEAA. Cells were fed every second day and split as described above for Caco-2 cells. For transmigration assays following splitting the cells were resuspended in 1 ml of medium and 70 µl of cell suspension was added to upper compartment of Transwell. Then

200 µl of medium was added to the upper compartment and 800 µl of medium to the lower compartment. The cells were incubated on filters for 4 days with daily medium change.

Human DC were cultivated in RPMI 1640 medium supplemented with 10% FCS, 100 µg/ml penicillin and streptomycin, 25 ng/ml GM-CSF and fresh medium was added every second day.

2.2.2. Isolation of human monocytes from blood and differentiation by IL-4/GM-CSF

20 ml of blood were layered on top of 20 ml of LymphoSep medium and centrifuged at 800xg for 40 min (no brakes). During that time Percoll gradient was prepared by mixing (per 20 ml of blood) 15 ml of Spinner medium, 13.5 ml Percoll and 1.5 ml of Hank's buffered salts solution (HBSS). Gradient was centrifuged at 10000xg 12 min. After centrifugation of blood leukocyte bands were collected, washed 3 times with Spinner medium (each centrifugation 6 min, 700xg and two times 500xg) and layered on top of Percoll gradient. Centrifugation was then performed 800xg for 35 min (no brakes). Peripheral blood mononuclear cells (PBMC) band was collected and washed twice with Spinner medium (each centrifugation for 6 min, at 600xg and at 500xg). The isolated cells were left to adhere for 1 hour in 6-well plates with approximately 10^7 cells per well in 2 ml of RPMI 1640 medium with FCS 10 % and 100 µg/ml penicillin and streptomycin. Cells were washed with warm PBS without Ca^{2+} and Mg^{2+} 5 times. Subsequently, 2 ml of RPMI 1640 medium with FCS 10%, 100 µg/ml penicillin and streptomycin, 100 ng/ml IL4 and 25 ng/ml GM-CSF were added. Medium was exchanged every second day and after 5-7 days cells were assessed for CD14 loss and expression of CD11c by using flow cytometry. After differentiation the cells were kept in culture not longer than 4 days.

2.2.3. Maintaining Caco-2 cell line on Transwell filters

The filters were pre-cooled for 1 hour at -20°C, then placed upside down in a sterile box with the lid and ice-cold suspension of extracellular matrix proteins (ECM) was added to cover the filter and left for 2 hours. After 2 hours the suspension of ECM was removed and the filters were dried. Caco-2 cells were split from the 75 cm^2 flasks as described above and the pellet was resuspended in 8 ml of fresh medium. 1 ml was used for further passages and an additional 1 ml of this suspension was then used for 24 filters after appropriate dilution (to each filter 100 µl of medium was added, so medium was added to a final volume of 2.4 ml), while the filters were left upside down. The box with filters was placed in the 37°C incubator with 5% CO_2 overnight. The following morning the filters were placed in their normal upright orientation into a 24-well plate. Medium was added to the upper and lower compartments of the Transwell filter. The cells were grown on the filters for 14 days. During this time the medium was exchanged every second day.

2.2.4. Maintenance of the intestinal barrier model

Dendritic cells were added to the basolateral side of the filters confluent with Caco-2 cells after 2 weeks of cultivation. In some experiments, DC were labeled with carboxyfluorescein succinimydil ester (CFSE) before adding to the filters. For this, the DC were centrifuged and resuspended in 2 ml of RPMI1640 medium without FCS. CFSE was then added to a final concentration 5 µM for 10 minutes (RT). Subsequently, the cells were washed twice with D-PBS without Ca^{2+} and Mg^{2+} but with 20% FCS. Then the DC were resuspended in appropriate amount of 1:1 DMEM:RPMI1640 mixture and added to the upper compartment of the Transwell filter system.

2.2.5. Infection procedures and viability assay

For infection experiments 10^6 cells such as THP-1 and DC (non-adherent cells) were seeded into each well of 12-well plates in 1.5 ml of medium. Bacteria were added in the desired MOI for 4 or 5 hours. Then the cells were centrifuged, washed once with PBS, resuspended in 100 µl PBS, and then propidium iodide (PI) was added to a final concentration of 10 µg/ml and incubated 5 minutes at room temperature in the dark. The cells were subsequently analyzed using a FACSCalibur flow cytometer employing CellQuestPro software.

2.2.6. Apoptosis and necrosis assays

The percentage of apoptotic cells was assessed using anti-annexin V antibodies followed by additional flow cytometric analysis (see **2.2.18**). The percentage of necrotic cells was estimated using a Cytotoxicity kit following the manufacturer's instructions. Briefly, following infection 50 µl of the cell suspension were added to a 96-well plate (3 wells per probe) and lysed using provided lysis buffer at 37°C for 40 min. Cell supernatants were prepared by centrifugation and added to the wells of a fresh 96-well microtiter plate (50 µl, 3 wells for each sample). The substrate for lactate dehydrogenase (LDH) was then added for 30 min, and the reaction stopped by adding Stop Solution. The LDH levels were measured colorimetrically at 490 nm using a Molecular Devices plate reader. After the data was acquired from experimental wells the negative control value was subtracted. To relate the LDH results to the number of cells, the ratio between [OD_{490} cells supernatant/OD_{490} cell lysates] was calculated for each experimental condition.

2.2.7. Cytokine secretion assessment

For the assessment of cytokine secretion by infected DC we applied the RayBio Inflammation Array. DC were infected with EPEC, C600, or EcN for 2 hours with a MOI of 10 in a 6-well plate (2 ml of medium in each well), washed, and fresh medium with antibiotics was

added and the incubation was continued for 16 hours. The contents of each well were centrifuged at 400xg for 4 min, the supernatants were taken and again centrifuged 12000xg for 5 min and filtered through 0.45 µm pore size filters. The assay - basically a sandwich ELISA - was then performed according to the manufacturer's instructions. Briefly, 1 ml of supernatant was added to a membrane pre-coated with antibodies to the cytokines under investigation (2 spots for each cytokine), then second and third antibodies were added with washing intensively after each step. The third antibodies were conjugated to horseradish peroxidase (HRP) that enables detection and quantification with a Lumi-Imager (Mannheim Boehringer). Obtained background values were subtracted to get final results for signal intensity which enable to make a conclusion about relative expression of given cytokine in different conditions.

2.2.8. Transmigration assay

HBMEC cells were seeded into the upper compartment of 3 µm pore size Transwell filter inserts and 800 µl of medium were added to the lower compartments. Cells were grown for 3 days with medium changes every day. THP-1 cells or DC were infected for 2 hours in MOI of 10 with EPEC, EcN or C600, and then gentamycin was added to final concentration 100 µg/ml for 4 hours, cells were centrifuged and placed in the upper compartment of the filter insert for 2, 6, or 18 hours. Then cells from the lower compartment were counted using a Coulter Counter (Beckton Dickinson).

2.2.9. Phagocytosis assay

Bacterial cells transformed with the *pVLT33-GFP* plasmid were inoculated in 1.5 ml of medium and grown overnight at 37°C. Overnight cultures were diluted 1:100, let to grow for 3 hours at 37°C with shaking, then IPTG was added at final concentration 0.5 µM. Bacteria were grown for 3 more hours, centrifuged at 5000xg for 5 min, counted, and THP-1 cells or

DC were infected with a MOI of 50 for 1 hour. Cells were then fixed with 4% PFA and flow cytometric analysis was performed. Uptake index was calculated as was suggested by Pils *et al*., by multiplying amount of GFP-positive cells by mean fluorescence (Pils *et al.*, 2005).

2.2.10. Counting of bacterial and eukaryotic cells

Eukaryotic cells and bacteria were quantified using a Coulter Counter (Beckton Dickinson) or counting chamber, respectively. Eukaryotic cells were diluted 1:40 in 8 ml of Isoton buffer and calculation was repeated 3 times. For the quantification of bacterial cells 10 µl of cell suspension (for overnight bacterial cultures – diluted 1:100) were added to the counting chamber between the grid surface and a coverslip tightly pressed to each other before adding the cell suspension. Then number of cells in 4 big squares was calculated and the average count per 1 square was determined. The number of bacterial cells per 1 ml was then calculated using the following relation:

$$\text{Cell count per 1 square} \times \text{dilution factor} \times 1.25 \times 10^6$$

2.2.11. OD_{600} estimation

1 ml of bacterial suspension was transferred into a cuvette and analysed with a Beckman DU640 spectrophotometer.

2.2.12. Bactericidal activity assessment

Microcin production was first tested on agar plates containing 0.2 mM dipyridyl to reduce the available Fe-ions. For this after overnight growth of a microcin producing strain (and a microcin-negative mutant as control) in spots (5-8 mm in diameter) tested strain was overlaid in 2.5 ml of soft agar (0.8%) with 0.2 mM dipyridyl, and the presence of growth-inhibition

zones was assessed after 16 hours. Strains that were found to be affected were further subjected to co-incubation tests. For this the microcin producing strain (and mutant as a control) was mixed 1:1 (10^7 cells of each strain) with the particular strain under study or the studied strain was grown by itself for 4 hours in 1.5 ml of RPMI medium with L-glutamine. Serial dilutions of the content of each well were plated on agar plates containing 30 μg/ml of kanamycin. Colonies were counted after 12 – 18 hours.

2.2.13. Bacterial transformation by electroporation

Strains were grown overnight, then diluted 1:50 and incubated with aeration until the OD_{600} reached 0.6 – 0.8. The cells were placed on ice for 5 min, centrifuged at 5000xg for 5 min at 4°C and washed 5 times with cold sterile H_2O (all procedures were performed on ice). The cells then were resuspended in a volume of water approximately equal to the volume of bacterial cells and the DNA to be transformed was added (0.5 – 1 μg). The content of the reaction tube (Eppendorf) was then mixed well and transferred to a cold 2 mm cuvette. Then the bacterial suspension was subjected to electroporation with the following parameters:

Voltage	2500 V	Capacity	0.0025 F
Resistance	0.0201 Ohm	Time	5 msec

Immediately following the electroporation 1 ml of fresh medium was added to the cuvette and incubation was continued for 1 hour at 37°C. 100 μl were then plated on agar with the required antibiotics, and the rest of the cells was pelleted, resuspended in 100 μl of sterile water and plated.

2.2.14. Bacterial transformation using $CaCl_2$ method

For transformation by the $CaCl_2$ technique, the cells were grown as for electroporation, but after the initial pelleting, the cells were put on ice (and all following procedures were also performed on ice), washed once with ice-cold $CaCl_2$ (30 mM) with centrifugation at 5000xg for 5 min and resuspended in 400 µl of $CaCl_2$. Then 0.5 – 1 µg of DNA was added for 10 – 30 min. The cells were then heat-shocked for 2 minutes at 42°C, immediately placed on ice, and 1 ml of fresh medium was added. Samples were then incubated at 37°C for 1 hour and then plated onto selective medium.

2.2.15. Immunofluorescent staining and microscopy

For examination by immunofluorescence following the specific treatment adherent cells were immediately washed once with PBS and fixed with PFA (4% solution in PBS) for 20 min. Non-adherent cells were first placed onto poly-L-lysine treated glass (for acquiring poly-L-lysine glasses 100 µl of poly-L-lysine solution was added on top of glasses for 5 minutes, washed with ddH_2O and dried) in a 24-well plate for 30 minutes at room temperature in the dark, washed once with PBS, and fixed with 4% PFA in PBS for 20 minutes. The cells were then washed with PBS and glycin (0.2% solution in PBS) was added for 20 min. After washing with PBS 1% BSA solution in PBS was added for 20 min. Immediately after removing the BSA solution, primary antibodies diluted in 0.1% BSA solution in PBS were added for 1 hour. After 3 washes for 5 min each, secondary antibodies diluted in 0.1% BSA solution in PBS were added for 1 hour. Samples were then washed 3 times and mounted on glass with mounting medium. In those cases where Transwell filters were used, all the reagents were added both to the upper and lower compartments, antibodies were first dropped on parafilm and then the filters were placed on top of the liquid and a few drops were added to the upper compartment. After staining the filters were cut and mounted between glass slides and round 12 mm cover glasses.

2.2.16. Processing samples for scanning electron microscopy (SEM)

For processing samples for scanning electron microscopy (SEM) the co-culture system was prepared as usual, infections were performed with a MOI of 50 for 2 hours. When necessary, the supernatant of EcN (10 µl) was added to the upper compartment of the filter. Following termination of the infection the cells were washed twice for 5 min with D-PBS and 2% glutaraldehyde in PBS was added overnight at 4°C. Embedding was performed at room temperature. First the filters were washed with D-PBS three times for 10 min, and dehydrated using a graded series of ethanol (30 min 30%, 30 min 50%, overnight 70%). Subsequently 90% ethanol was applied for 30 min followed by 96% ethanol for 30 min. Finally 99.8% ethanol was added twice for 30 min each time. Filters were cut, dried under vacuum, sputtered with platinum and placed on grid. Pictures were acquired using a REM Leo 1530 VP. Fixation, picture acquisition and processing were performed by L. Greune (Institute of Infectiology, ZMBE).

2.2.17. Preparation of supernatants of EcN cultures

EcN was grown with shaking in standard medium until the OD_{600} reached 0.5 – 0.7. Bacteria were removed by centrifugation at 5000xg for 5 min. Another centrifugation step followed 12000xg 10 min. The supernatant carefully removed and subsequently filtered through 0.45 µm pore size filter and either frozen at -20°C or applied directly to the upper compartment of the Transwell filters. 10 µl of the supernatant were plated to ensure that the supernatants were not contaminated with residual bacteria.

2.2.18. Flow cytometry

For labeling of cells for the flow cytometry the following protocol was applied: $5x10^5$ – $1x10^6$ cells were centrifuged, washed once with PBS and resuspended in 500 µl of 1% BSA in

PBS (in case if anti-annexin V antibodies were used cells were washed once with binding buffer and resuspended in 70 µl of binding buffer). Antibodies were then added in the appropriate dilutions and incubated for 30 – 60 minutes in the dark at RT. The cells were then washed twice with PBS and then either resuspended in 200 µl of 1% PFA in PBS for further analysis, or in 500 µl of 1% BSA in PBS if secondary reagents are to be applied. Secondary reagents were added for 30 minutes, and the cells were again washed twice with PBS and resuspended in 200 µl of 1% PFA in PBS. Then the cells were monitored and evaluated by flow cytometer using a FACSCalibur (Beckton Dickinson) employing the CellQuestPro software.

2.2.18. RNA isolation

2.2.18.1. Isolation of RNA for qRT-PCR

For RNA isolation from THP-1 cells or DC 10^7 cells in 10 ml of tissue culture medium were infected with the desired MOI of the respective strains (usually a MOI of 10) for 2 hours, 100 µg/ml of gentamycin was added for 2 hours and then the RNA was isolated using a High Purity RNA Isolation Kit (Roche) according to the manufacturer's instructions. Briefly, cells were lysed and the lysate obtained was applied to a glass column to bind nucleic acids. Then DNAse H was applied to the column and, subsequently, the glass column was washed 3 times with ethanol-containing buffers and dried. Then 50 µl of water were added to each column and the residual bound RNA washed into an Eppendorf tube and either processed directly with reverse transcription or frozen at -70°C for further analysis.

2.2.18.2. Isolation of whole pool of RNA for micro-RNA array

Following infection the cells were centrifuged at 400xg for 4 min and then washed with PBS. Then 4 ml of TRI reagent (Sigma) was added and pipetted up and down for a few

minutes until the solution was no longer viscous. Cells and cell debris were removed by centrifugation at 9000xg for 10 min at 4°C and the supernatant was transferred into a fresh tube. Chloroform was then added to 20% (i.e. 0.2 ml chloroform for each 1 ml TRI reagent). Tubes were shaken and 'vortexed' vigorously for at least 15 seconds and left at room temperature for 10 min. Samples were centrifuged for 15 min at 9000xg at 4°C. The upper aqueous phase was transferred to a fresh tube and 0.5 volume of isopropanol was added (i.e. 0.5 ml isopropanol for each ml TRI reagent) and mixed immediately by inverting the tubes 5-8 times. Samples were incubated at room temperature for 5-10 min and then centrifuged at 9000xg for 10 min at 4°C. The supernatants were discarded and the samples were washed with 1 ml of 75% ethanol (RNAse free). The pellets were then dried, resuspended in nuclease-free water and kept at -70°C until further analysis.

2.2.19. Reverse transcription and quantitative RT-PCR

Reverse transcription was performed using the High Fidelity First Strand cDNA Synthesis Kit (Roche) and a thermocycler (Biometra). Briefly, 1 µg of RNA was taken for each reaction, mixed with 1.3 µl of poly-T primer (final concentration 1 µM) and water in a way that the final volume was 13 µl. The master mix were then prepared by calculating 7 µl for each reaction (4 µl buffer, 2 µl dNTPs, 0.5 µl RNAse inhibitors, 0.5 µl polymerase). The thermocycler program was the set using the following parameters:

Preheating	99°C	
Primer annealing	65°C	10 min
	1°C pause	
	Add Master Mix	
Extension	55°C	30 min
Denaturation	85°	5 min
cooling	4°C	as necessary

Samples were then frozen at -20°C for further analysis or processed immediately by qRT-PCT (Light Cycler, Roche). Samples preparation for qRT-PCR was performed on ice. Appropriate dilutions were prepared (in this study the following dilutions were used: 1:10, 1:20, 1:50, 1:100). The required amount of capillaries were prepared (for each sample: 2 for each primer of interest and 4 for housekeeping gene primer + negative control for each primer), to each 4 µl of water was added (and 6 µl to negative controls capillaries). Then 2 µl of each primer, 2 µl of the appropriate dilutions of DNA (for housekeeping gene all 4 dilutions, for others first 2 dilutions), buffer and polymerase were added, and samples were processed employing a LightCycler using the following program:

50 cycles:		
Denaturation	95°C	10 sec
Primer annealing	60°C	10 sec
Extension	72°C	12 sec
1 cycle:		
Melt	65°	15 sec
1 cycle:		
Cool	40°	30 sec

The primers used in that work are listed in table:

Name of the gene	*Primer pair*
IL10	left tga atg cta ccc caa ttg ct right ggc tgt gca ttg acc ttt c
IL12p35	left tct gtt tta gga agc cct gca right tgg aaa ccc ctt gcc ata gc
Glucose-6-phosphodehydrogenase	left gca aac aga gtg agc cct tc right ggc cag cca cat agg agt

Table 7. Primers used in the study.

3. Results

3.1. EcN has a direct effect on the viability of EPEC strains

EcN was tested for growth inhibition activities towards various *E. coli* strains. An initial antagonistic test assessed growth inhibitory activities of EcN by overlaying the tested bacteria in soft agar on top of agar with EcN or SK22D (microcin-negative mutant of EcN) grown in 1 cm spots. In this assay 13 of 32 tested strains showed growth inhibition zones around EcN but not SK22D spots. The inhibitory activities of EcN were further confirmed by plating and the susceptible strains were selected accordingly (Table 8).

E. coli strain	Inhibition by EcN microcins	*E. coli* strain	Inhibition by EcN microcins
EPEC E2348/69	+	EAEC 02/1850	-
EPEC Cigleris	+	EAEC Fu 297-1	-
EPEC 5274	+	EAEC Fu 214-4	-
ATEC 9812	+	EAEC Fu 154-2	+
ATEC B6	+	EIEC 99-10281	+
ATEC 0036/78	+	EIEC 99-10282	-
ATEC 0181-6/86	+	EIEC 02-10479	-
ATEC 660-79	+	EIEC 99-10280	-
ATEC 10459	-	EIEC 99-10283	-
ATEC 10419	-	EIEC 00-4745	+
ATEC 3431	-	EHEC 493/88	+
ATEC 11638	-	EHEC 5720/96	-
ATEC 15740	-	EHEC 2996/96	-
ATEC 15716	-	EHEC EDL933	+
ATEC 1972-1/85	-	*E. coli* B BL 21	-
EAEC 260	-	*E. coli* K12 C600	-

Table 8. Susceptibility of different *E. coli* strains to EcN microcins. Absence (-) or presence (+) of lysis zones on 2,2'-dipyridyl agar are indicated.

Examples of lysis zones after 18 hours of incubation are shown in Fig. 9. The diameter of the lysis zones never exceeded 1.2 mm (EPEC E2348/69) which might be attributed to the

instability of the microcins secreted by EcN. For many other strains the lysis zones were so subtle, that quantification was impossible.

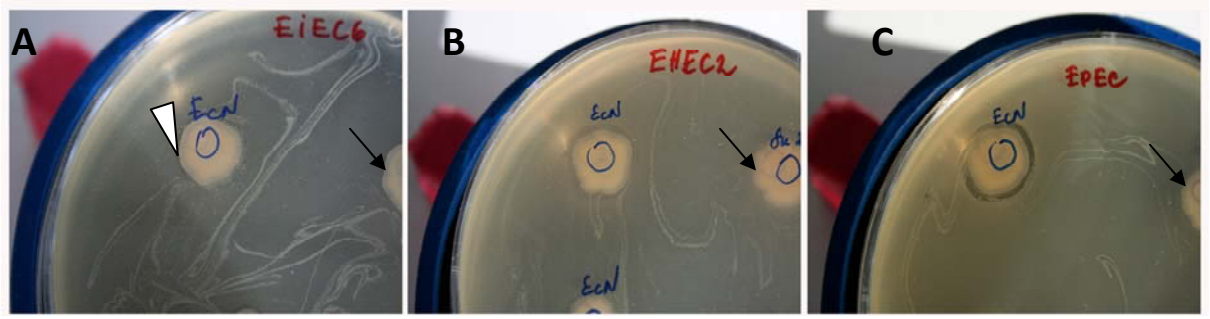


Fig. 9. Lysis zones around EcN spot after 18 hours growth of the selected strains. A – EIEC 99-10282, B – EHEC 5720/96, C – EPEC E2348/69. Part of SK22D spot is marked with an arrow. For the EIEC strain lysis zones was barely visible (arrowhead), for the EPEC strain the effect was the most profound.

Each of the tested strains was transformed with the pVLT33-GFP plasmid and then 10^7 cells were inoculated in 3 wells of a 12 well plate in 1.5 ml of RPMI 1640 medium without antibiotics. To two of the wells equal numbers of EcN or SK22D bacteria were added. To assess survival of the bacteria, after 4 hours of incubation serial dilutions from each well were plated on agar with 30 µg/ml kanamycin, incubated at 37°C overnight, then the resulting colonies were counted. Fig. 10 reflects the ratio between strains grown in the presence of EcN or SK22D and grown without the presence of other bacteria. The effect of EcN can be clearly attributed to the activity of the microcins H47 and/or M as the microcin-deficient strain SK22D has no inhibitory effect.

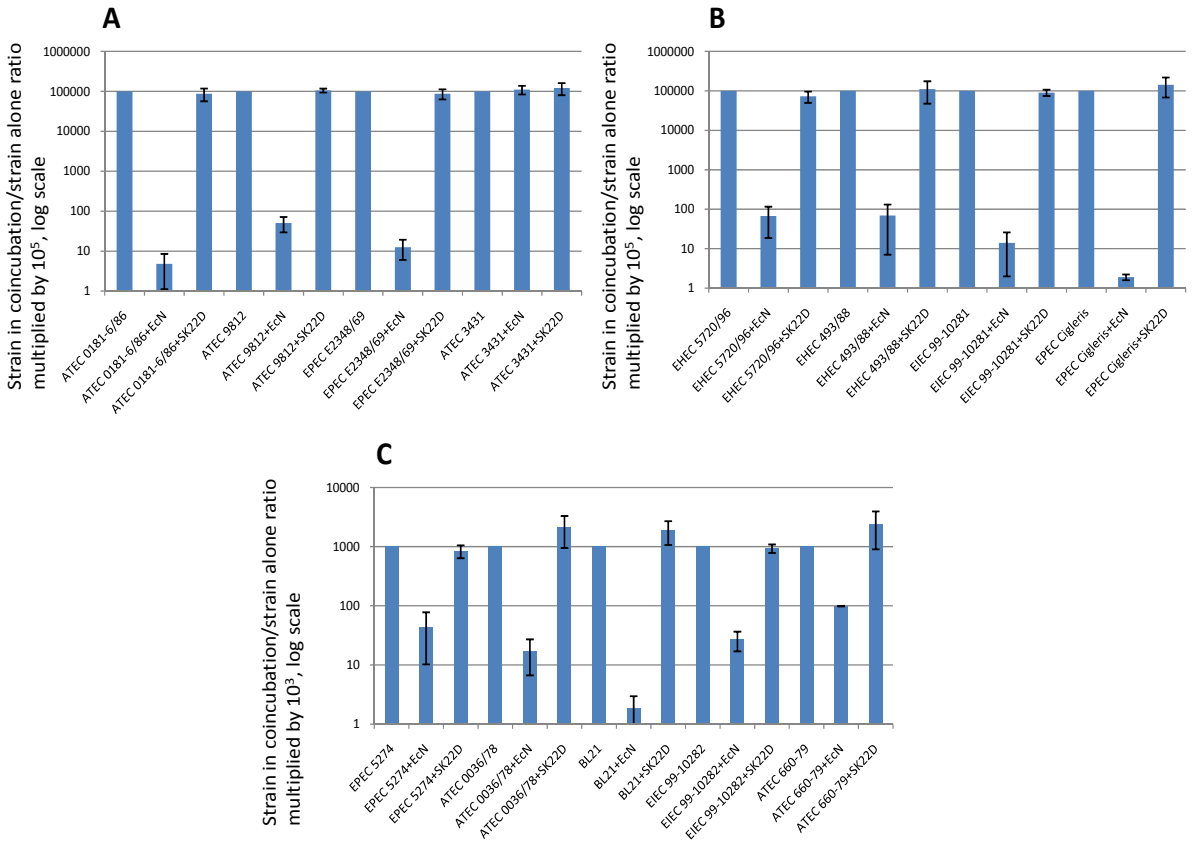


Fig. 10. Inhibitory effect of *E. coli* Nissle 1917 on the multiplication of various different *E. coli* strains. (Please note that a logarithmic scale has been used for the ratios of the [strain in co-incubation vs. strain alone] (hence a single strain is always 1) multiplied by 10^5 for ratios from $3x10^{-6}$ to $5x10^{-4}$ and for not affected ATEC 3431 strain shown for comparison (A and B) or 10^3 for strains with ratios from 0,001 to 0,03 (C)). For each strain experiment was performed at least 3 times in triplicate, error bars reflect standard error.

In general, **all typical EPEC** strains used in this study were susceptible to microcin activities, while **all enteroaggregative** *E. coli* strains investigated proved to be resistant. Among ATEC, EHEC, and EIEC pathotypes both susceptible and resistant strains were identified. However, the number of strains investigated in this assay does not allow for a general conclusion such that all typical EPEC strains are susceptible to EcN microcins. Among laboratory strains tested, the *E. coli* B strain was found to be susceptible (*E. coli* BL21) whereas the K-12 strain was found to be resistant (*E. coli* C600).

3.2. The ATEC B6 strain is able to form aggregates and is affected by EcN microcins irrespective of aggregates formation

After 2 hours of incubation in RPMI 1640 the ATEC B6 strain was found to form aggregates which were visible for up to 8 h (37°C, 5% CO_2). To investigate whether EcN microcins might be able to kill also aggregated bacteria, we added EcN either simultaneously or 2 hours after adding the *E. coli* B6 strain. In both cases the proliferation of ATEC B6 was affected by EcN microcins and aggregates were either not formed or disappeared during the monitoring period of 4 hours (Fig. 11). Although occasionally multicellular structures could be observed when ATEC B6 was cultivated prior to the addition of EcN, these included much less bacteria and were not bound as tightly. Representative results of co-incubation experiments are shown in Fig. 12.

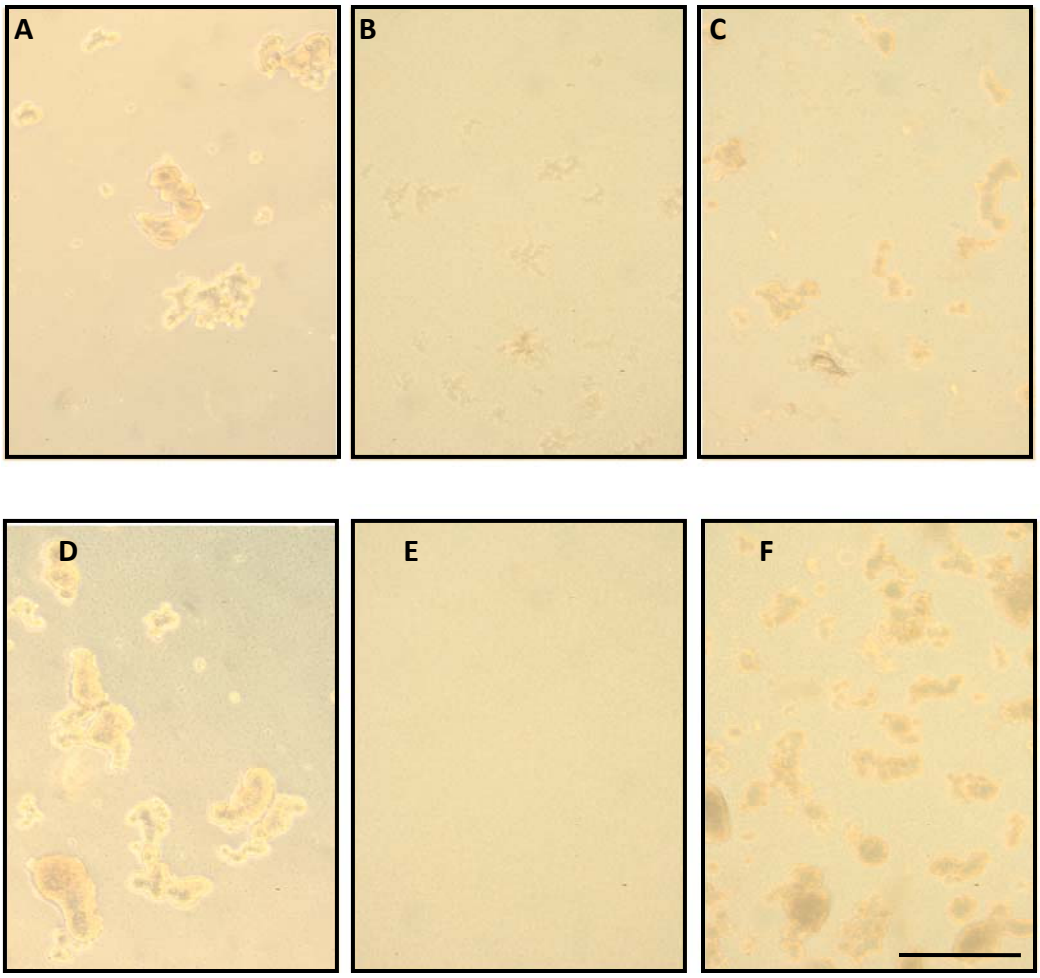


Fig. 11. Aggregates formation by ATEC B6 strain: A) after 6 hours at 37°C, B) after 2 hours incubation at 37°C with the subsequent addition of EcN, or C) SK22D for 4 hours, D) after 4 hours at 37°C, and E) after 4 hours incubation together with EcN or F) SK22D. Experiment was performed 3 times in triplicate. Scale bar 1mm.

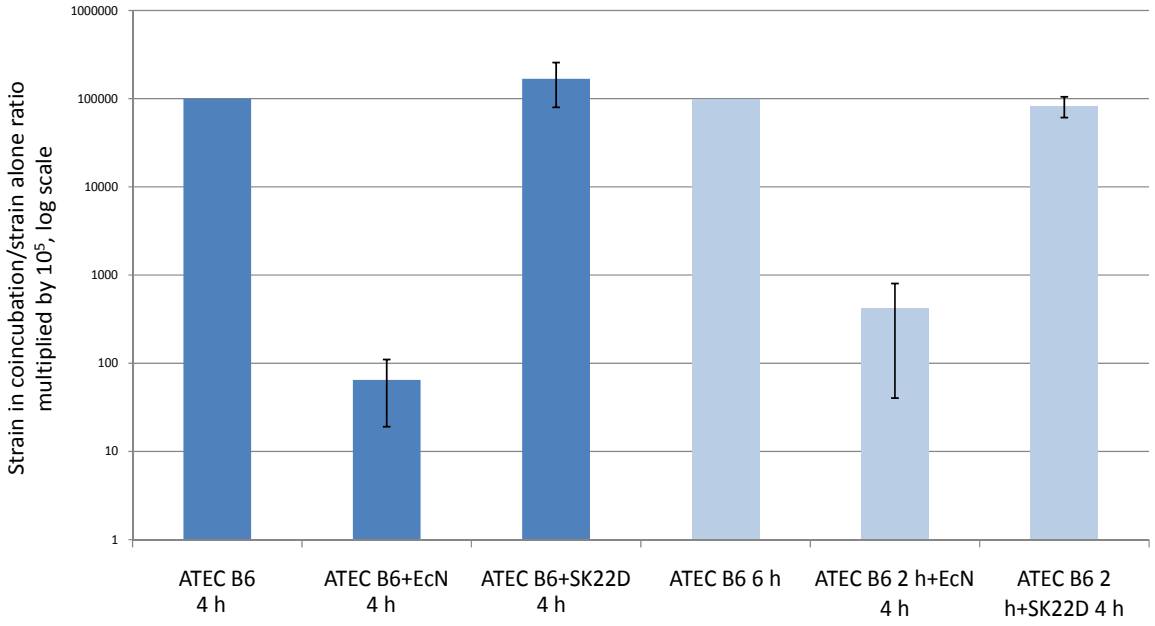


Fig. 12. Inhibitory effect of EcN on the growth of the biofilm-forming strain ATEC B6. (Please note that a logarithmic scale has been used for the ratios of the [strain in co-incubation vs. strain alone) (hence a single strain is always 1) multiplied by 10^5). The experiments were performed 3 times in triplicate each. The error bars reflect standard error.

3.3. The EPEC E2348/69 strain induces pedestal formation also on immune cells such as monocytic THP-1 cells and human DC

THP-1 cells (appr. 10^6) in 2 ml of RPMI 1640 medium in 12-well plates were infected with EPEC E2348/69 bacteria with a MOI of 10 and incubated at 37°C 5% CO_2 for 4 hours. The contents of each well were applied to poly-L-lysine treated coverslips to attach the cells, which were washed, fixed with PFA, and stained with anti-Tir antibodies, Texas Red conjugated phalloidin, and DAPI. Alternatively, GFP-expressing bacteria were used.

Subsequently, the samples were analyzed by immunofluorescent microscopy using a Zeiss Axiophot fluorescence microscope.

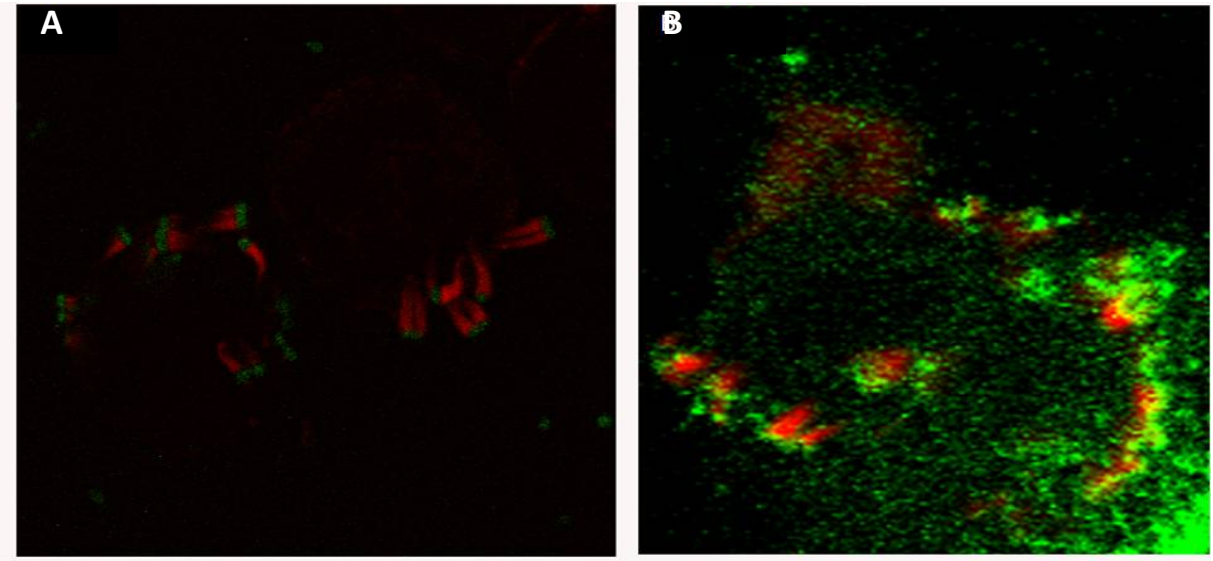


Fig. 13. Pedestal formation after infection with EPEC E2348/69-GFP on THP-1 cells (stained with phalloidin-Texas Red conjugate for polymerized actin - red; bacteria expressing GFP - green) (A) and after infection with EPEC E2348/69 on human DC (stained with phalloidin-Texas Red conjugate (red) and anti-Tir antibodies - green) (B). Scale bar 1 µm.

3.4. The E2348/69 EPEC strain affects the viability of THP-1 cells

10^6 THP-1 cells in 2 ml of RPMI 1640 medium were seeded into 12-well plates and bacteria were added with a MOI of 10 for 4 hours or alternatively with a MOI of 20 for 5 hours. The contents of each well were then transferred into the Eppendorf tube, centrifuged at 1000xg for 5 min, washed once with PBS, resuspended in 100 µl PBS, stained with PI and evaluated by flow cytometry. After 4 hours of infection with EPEC E2348/69 and several TTSS effector mutants only the wild type strain caused a significant decrease in cell viability, while all the mutants tested (Δ*escN*, *ΔsepZ*, *ΔeaeA*, *Δtir*, *ΔespD*, Δ*espB*, Δ*espF,* and Δ*map*) failed to do so.

As it was impossible to conclude which specific factor might be responsible for the oberved effect, we also investigated cell death of THP-1 cells after 5 hours of incubation to see whether the TTSS mutants listed above might only postpone but not abolish cell death. Fig. 14 represents the percentage of cells that excluded PI and were therefore not affected. Surprisingly, under these conditions we observed that with both *tir* and *eaeA* (intimin) mutants the lethality rates were comparable to those of the wild type strain and only for the TTSS-negative (*ΔescN*) mutant and the *esp*D mutant the ability to cause cell death was abolished. Infection with the *Δsep*Z as well as with Δ*espB*, Δ*espF,* and Δ*map* resulted in intermediate levels of PI inclusion. Interestingly, it had been reported that many events triggered by EPEC such as pedestal formation are postponed but not completely abolished for *sep*Z mutants (Devinney *et al.*, 2001). The tendency of these TTSS mutants for a reduction in THP-1 cell viability are clearer when compared to the viability of cells infected with EcN which was used as a control of dying just because of nutrient depletion. Therefore, it should be concluded that the factor(s) responsible for THP-1 cell death are/is secreted by the TTSS and that EspD is directly or indirectly involved in the viability decrease observed in these experiments.

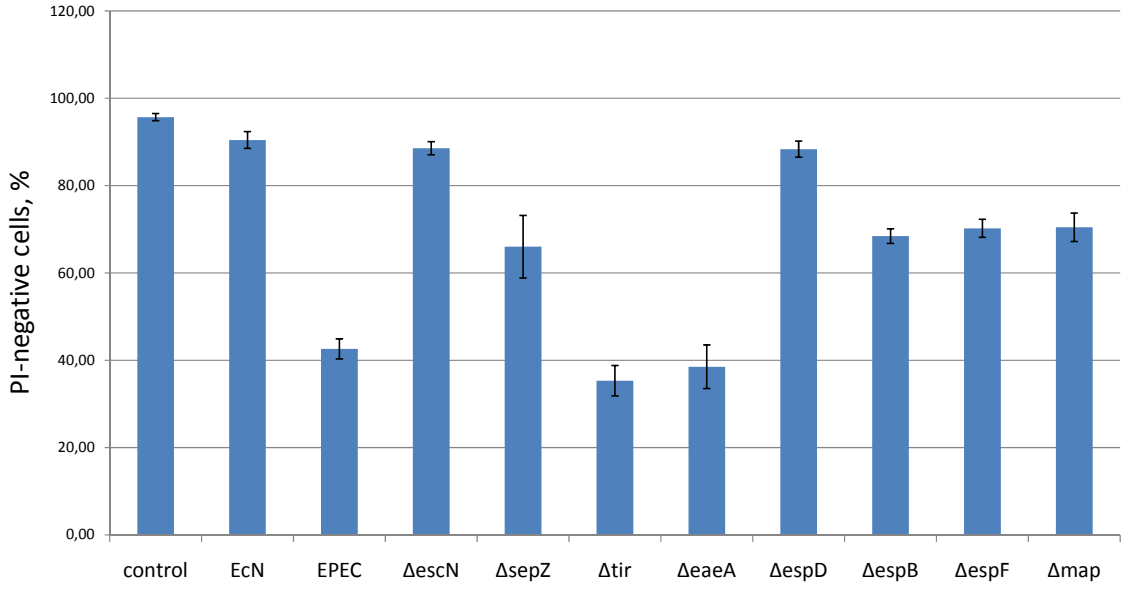


Fig. 14. THP-1 cell death as monitored by PI staining after infection with EPEC and selected EPEC mutants. The percentage of cells that excluded PI (hence alive) is shown. THP-1 cells were infected for 5 hours with a MOI of 10. The experiments were performed 5 times in triplicate each. Error bars represent the standard error.

To investigate whether the observed effects might be due to induction of apoptosis after infection the cells were incubated with anti-annexin V antibodies. Fig. 15 gives the percentage of annexin V positive cells as identified by flow cytometry.

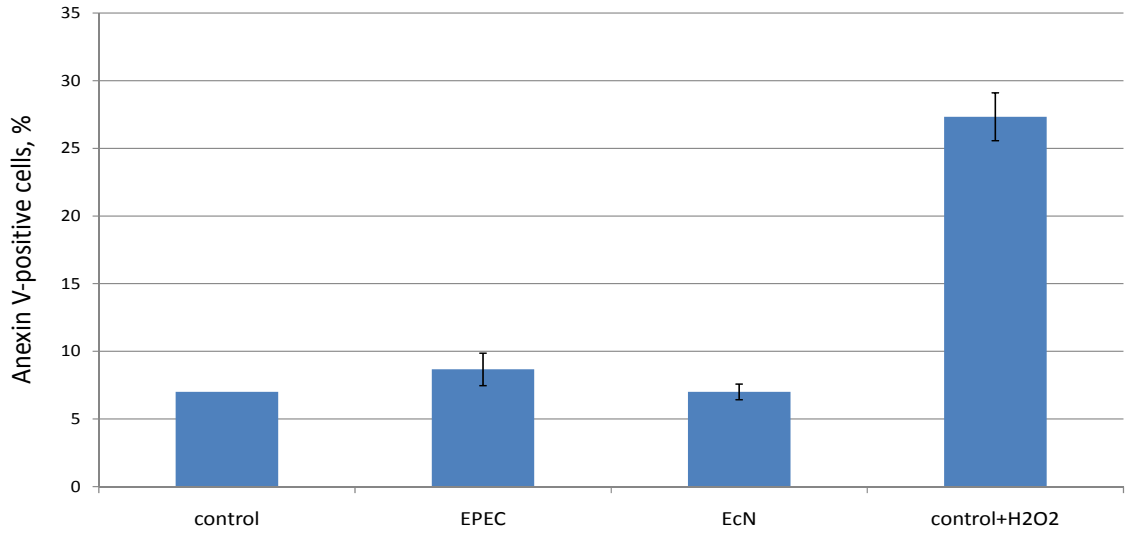


Fig. 15. Percentage of apoptotic THP-1 cells after infection with EPEC or EcN. Untreated cells were used as negative controls, cells treated with 30 mM H_2O_2 were used as positive controls. Apoptosis was assessed by flow cytometry using anti-annexinV antibodies. The experiment was performed in triplicate. Error bars represent standard error.

Next, the amount of lactate dehydrogenase (LDH) in the supernatants of infected cells was determined using a Cytotoxicity kit (Sigma) to evaluate whether cell lysis occured following EPEC infection. This would point to a contribution of mechanisms resulting in necrosis as a possible cause for cell death. Results from these experiments are depicted in Fig. 16. These experiments clearly showed that lysis does occur as the amount of LDH in the supernatnat of EPEC infected cells was higher than in uninfected control cells. However, the LDH levels were still lower than was expected, and neither the *eae* (intimin) mutant nor the *sep*Z mutant were causing higher LDH release than control or EcN infected cells. Δ*espB*, Δ*espF* and Δ*map* cause the same LDH release as the wild type strain. Interestingly, the Δ*tir* mutant caused the highest level of LDH release which was twice as high as in control cells.

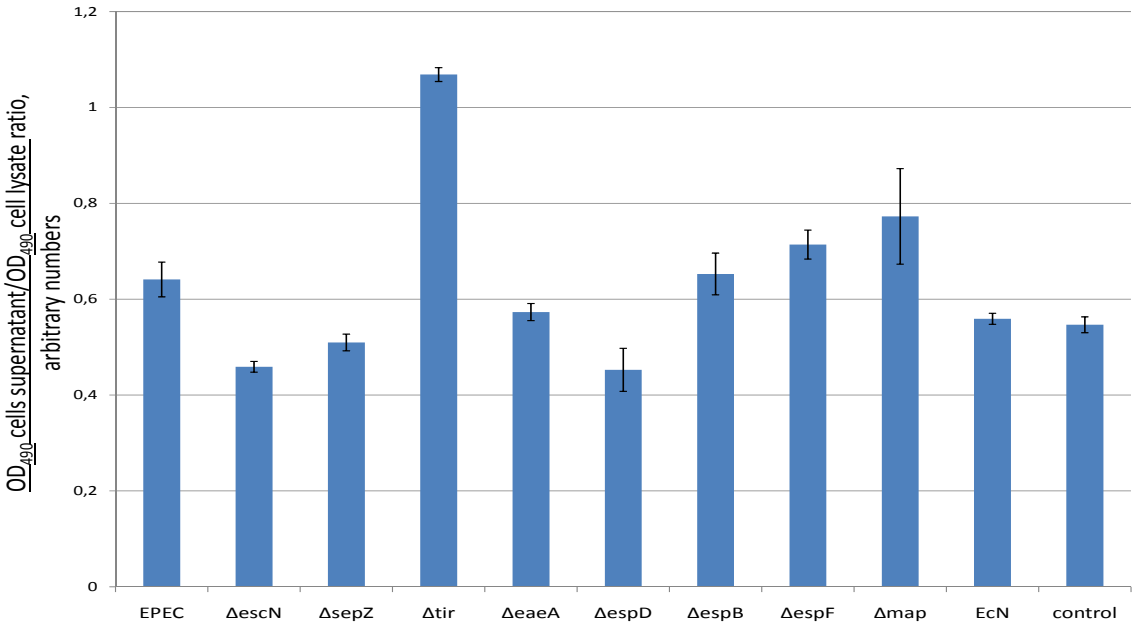


Fig. 16. LDH release by THP-1 cells infected for 7 hours with EPEC, its mutants, and EcN. Bars reflect the ratio of [OD_{490} cells supernatant/OD_{490} cell lysate] for each set-up in arbitrary numbers. Therefore higher numbers indicate that more cells have been lysed. Uninfected cells have been used as a control. The experiments were performed 3 times in triplicate each. Error bars represent the standard error.

THP-1 cells were further studied microscopically to detect potential phenotypic differences in infections with different strains. In Fig. 17 micrographs obtained 5 hours post-infection are shown. For *map*, *espF*, *espB,* and *espD* mutants very distinct phenotypes were observed as most of the bacteria were tightly bound together forming large conglomerats that also included THP-1 cells. Almost no eukaryotic cells were found not to be part of such a multicellular structure. The *Δtir* mutant showed a similar phenotype, only that the conglomerats were smaller including 5 – 10 cells, and that also some isolated cells were found. In this setup, the wild type strain was not different from an *escN* mutant. Both strains did not induce multicellular structures, although THP-1 cells were found in groups of 3 – 8 cells. EcN infected cells were not different from uninfected cells used as controls in that these did not induce formation of any multicellular aggregates.

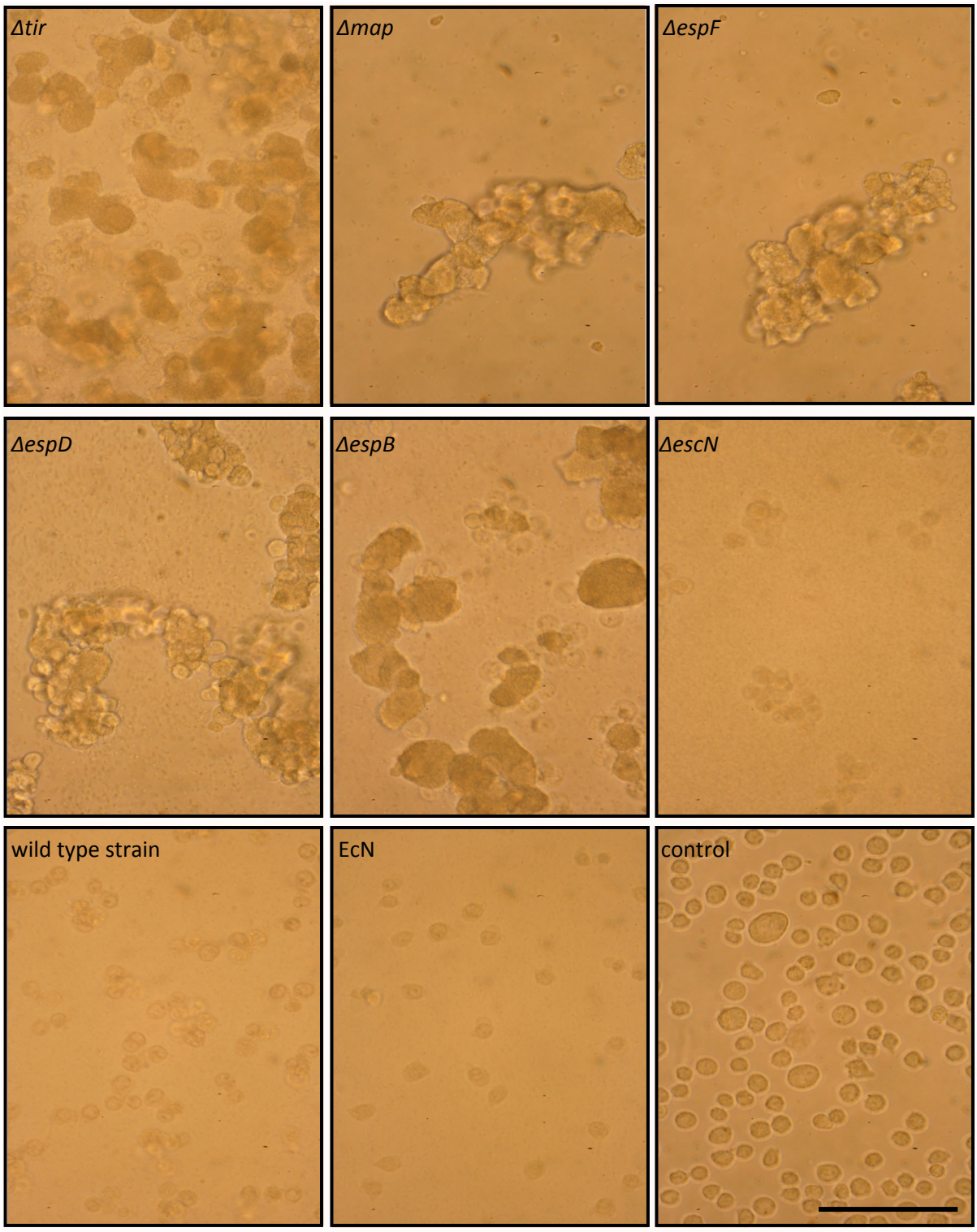


Fig. 17. THP-1 cells infected with different EPEC mutants and EcN for 5 hours. As a control uninfected cells were used. The experiments were performed 5 times in triplicate each. Error bars represent the standad error. Scale bar 1 mm.

3.5. EcN prevents EPEC-induced cell death of THP-1 cells in co-infections

THP-1 cells were infected with different bacteria with a MOI of 10 for 4 hours (in case of co-infection experiments both strains were used with a MOI of 10). Cells were then analyzed for their permeability for PI. The percentage of live intact cells (excluding the dye) after bacterial infections is given in Fig. 18. Only wild-type EcN but not the microcin-negative mutant SK22D was able to abolish the negative effects from EPEC infections. This indicates that the rescue is based on the bactericidal activity of H47 and/or M microcins. Therefore, one can conclude that the EcN microcins exert a strong bactericidal effect on EPEC that is sufficient to prevent this pathogen from causing pathological processes characteristic for its infection process.

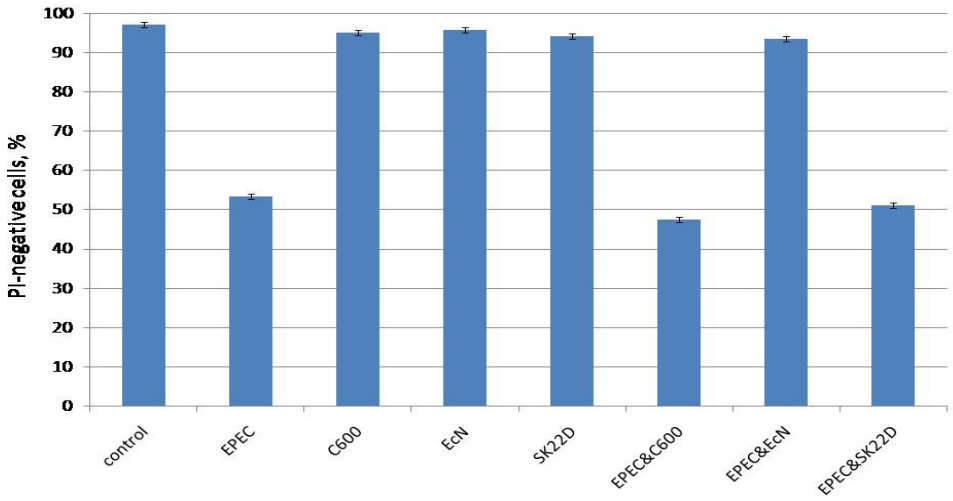


Fig. 18. THP-1 viability in co-infections with two bacterial strains. The percentage of cells that excluded PI is shown. The infection continued for 4 hours. The experiments were performed 3 times in triplicate each. Error bars represent the standard error.

3.6. Influence of *E. coli* K-12 strain C600 on the adhesion of THP-1 cells and DC to solid surfaces

Infection with non-pathogenic *E. coli* K-12 C600 bacteria was found to enhance the attachment of DC and THP-1 cells to various surfaces while cells infected with pathogenic EPEC bacteria more often detached. EcN-infected cells behaved as the uninfected cells. Enhanced adhesion was observed to all tested surfaces, namely 24-well plates for the cell culture, FCS or ECM treated coverslips, and untreated glass surfaces. To further investigate this effect THP-1 cells were seeded either in 24-well plates for tissue culture or in 24-well plates with untreated glass coverslips on the bottom of the wells. Bacteria were added to THP-1 cells with a MOI of 10 for 4 hours. Then the supernatant with non-adherent cells was removed and adherent cells were washed gently with warm PBS (37°C) followed by immediate fixation with 4% PFA. Then six micrographes were taken for each well in randomly chosen different areas (Canon 350D camera; 14 x magnification). The average number of cells per field of view was determined. Fig. 19 depicts the attachment of THP-1 cells to culture dishes and to glass coverslips (average amount of attached cells per field of view).

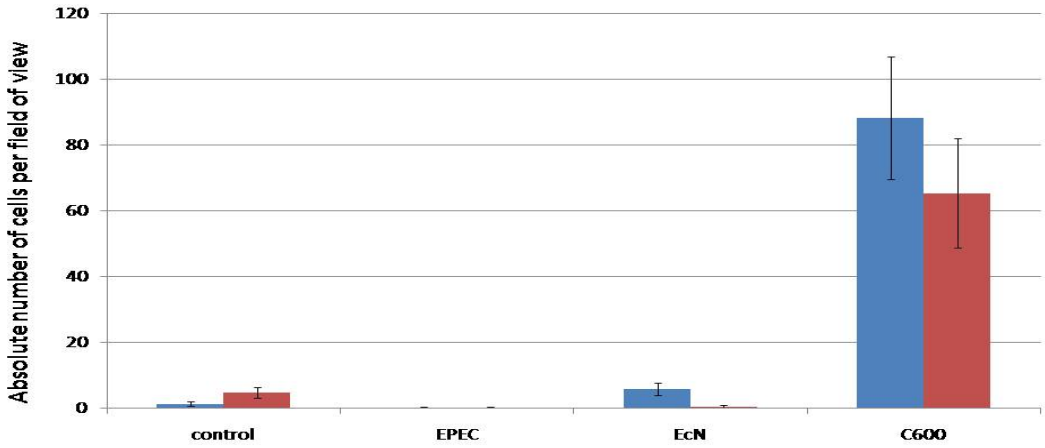


Fig. 19. Adhesion of THP-1 cells to tissue culture 12-well plates (blue) or to untreated glass coverslips (red) after 4 hours of infection with indicated strains. Average amount of cells per field of view is shown. The experiments was performed 3 times in triplicate each. Error bars represent the standad error.

As THP-1 cells are normally non-adherent, the difference between uninfected control and EPEC-infected cells seems minor, however, for EPEC-infected cells the average number of adherent cells per field of view was less than 1. For non-infected cells a few cells (never less than 2 per field of view) always adhered to the surface. For *E. coli* C600-infected THP-1 cells at least 50 cells could be seen per field of view.

For EPEC infections the detachment of HeLa cells has been observed previously (Marques *et al.*, 1995; Crane *et al.*, 1999). This effect is most probably connected to the ability of EPEC to decrease the cell viability as non-viable cells apparently lose their adherence phenotype.

To further investigate this effect we performed transmigration experiments to assess whether the migration capabilities of DC and THP-1 cells might also be affected following infection with different *E. coli* strains. For transmigration experiments human brain microvascular endothelium cells (HBMEC) were used as this is an easy to handle cell line which has maintained all the main characteristics of endothelial cells and presumably reflects *in vivo* properties of an endothelial layer on Transwell filters. Then THP-1 cells or DC were infected for 2 hours with a MOI of 20 and gentamycin (100 µg/ml) was added for 2 additional hours. Subsequently, the cells were harvested by centrifugation (400 g for 4 min), resuspended in 200 µl of fresh medium with antibiotics and then added to the confluent

HBMEC layer (to the upper compartment) for 14 more hours. Cells in the lower compartments were quantified with a Coulter Counter. Fig. 20 and Fig. 21 reflect the number of cells in the lower compartment of the Transwell filter system. In the case of DC only cells infected with *E. coli* C600 showed alterations in transmigration as shown by an increase of cells in the lower compartment from 30,000 (± 2,000) to 42,000 (± 4,000). For THP-1 cells, however, transmigration of cells through the endothelial layer was not significantly altered after infection.

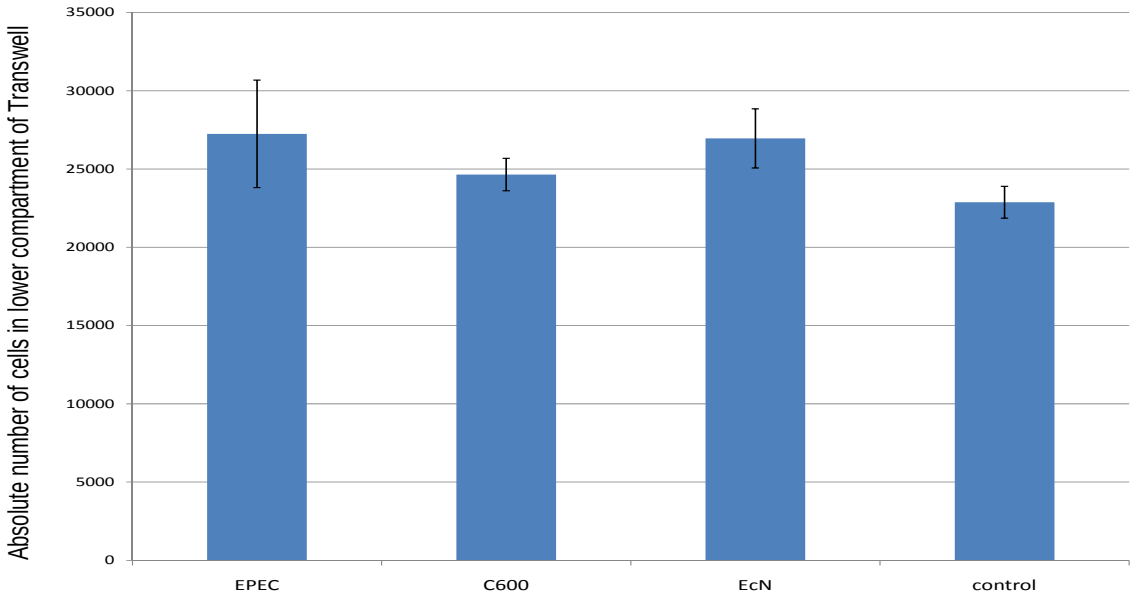


Fig. 20. Transmigration of THP-1 cells infected with bacteria through a HBMEC layer on Transwell filters. The experiments were performed 3 times in triplicate each. Error bars represent the standad error.

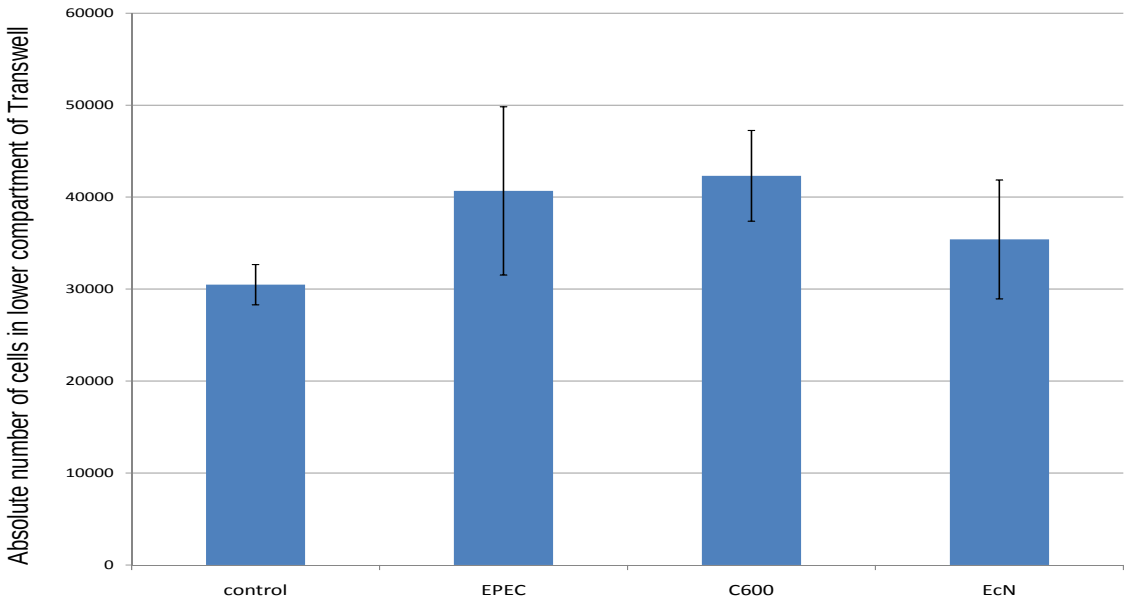


Fig. 21. Transmigration of THP-1 cells infected with bacteria through a HBMEC layer on Transwell filters. The experiments were performed 3 times in triplicate each. Error bars represent the standad error.

3.7. Global transcriptome analysis of DC and THP-1 cells after infection with EcN and EPEC

Transcriptom analysis using DNA-microarray technology was performed with DC and THP-1 cells following infection with EPEC and EcN (all analysed against control uninfected cells). Infection was performed for 2 hours with a MOI of 10, then 100 µg/ml gentamycin was added for 2 hours, followed by the isolation of total RNA. The RNA was monitored for purity and its concentration was determined. A microarray chip (Human Genome U133 Plus 2.0 Array) was used to assess the gene expression profile. Data were then analysed for statistical significance employing GeneSpring GX Software. All results with P-values higher than 0.05

were not taken into account. From statistically significant data only values indicating a change of expression 2-fold or higher were considered for further analysis.

Based on these parameters, four groups of genes were identified: *i*) genes up-regulated in both EcN and EPEC infected cells, *ii*) genes down-regulated in both EcN and EPEC infected cells, *iii*) genes up-regulated for EPEC but down-regulated for EcN, and *iv*) genes up-regulated for EcN but down-regulated for EPEC infected cells. For DC most of the genes altered in transcription were regulated with a similar trend, however, often to a different extent. Seven genes were shown to be down-regulated in EcN and up-regulated in EPEC and 41 genes – vice versa (Fig. 22). For THP-1 cells more genes identified during the microarray analysis passed statistical validation, and most of them also showed the same regulation for EPEC and EcN infected cells (Fig. 23).

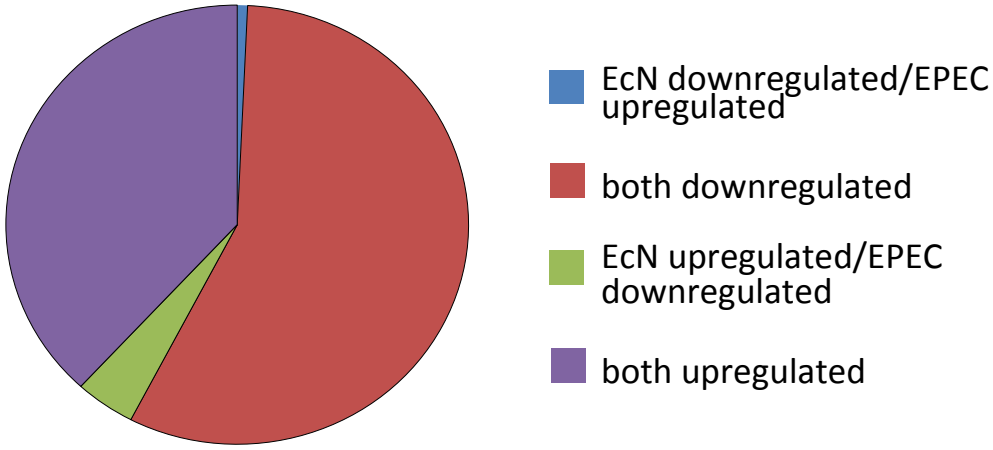


Fig.22. Groups of genes in DC affected in their regulation by infection with either EcN or EPEC bacteria.

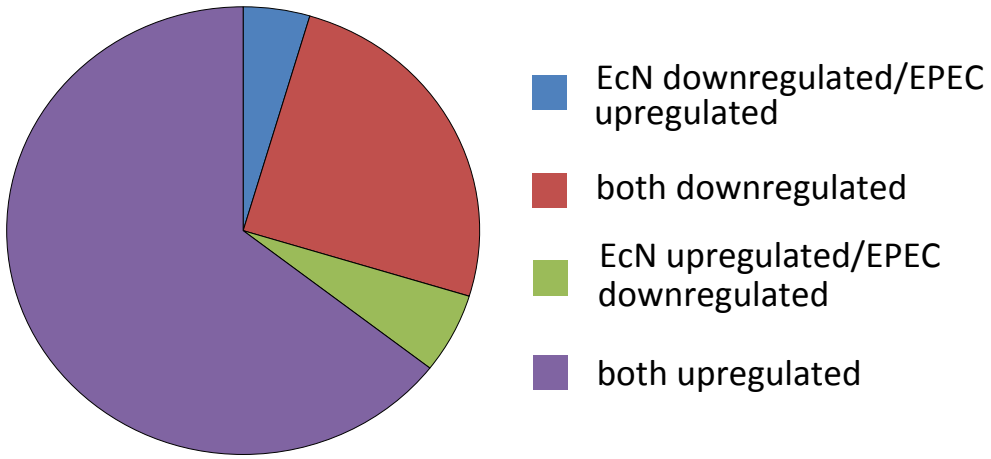


Fig. 23. Groups of genes in THP-1 affected in their regulation by infection with either EcN or EPEC bacteria.

The most interesting genes were selected for further analysis. Mainly genes regulated differently in infection with different strain were selected (Tables 9 and 10).

Gene Symbol	*Full name of the gene*	*Fold change EPEC*	*regulation EPEC*	*regulation EcN*	*Fold change EcN*
Immune response					
BIC	miRNA-155 precursor bic	8.9	up	up	11.7
CCBP 2	chemokine binding protein 2	6.5	down	up	2.3
CCL2	chemokine (C-C motif) ligand 2	3.3	up	up	8.2
CCL5	chemokine (C-C motif) ligand 5	2.0	up	up	4.5
CCL14	chemokine (C-C motif) ligand 14	1.8	NA	up	2.2
CCL20	chemokine (C-C motif) ligand 20	688.8	up	up	1882.2
CCL22	chemokine (C-C motif) ligand 22	1.2	NA	up	2.7
CXCL2	chemokine (C-X-C motif) ligand 2	3.4	down	up	10.7
CXCL3	chemokine (C-X-C motif) ligand 3	1.5	NA	up	6.8
CXCL5	chemokine (C-X-C motif) ligand 5	1.2	NA	up	2.3
CXCL11	chemokine (C-X-C motif) ligand 11	33.7	up	up	15.1
CXCR4	chemokine (C-X-C motif) receptor 4	3.6	up	up	3.0
XCL1 /XCL2	lymphotactin1/lymphotactin2	9.5	up	up	3.3

IL1A	interleukin-1A	2.0	NA	up	13.2
IL1B	interleukin-1B	3.8	up	up	6.1
IL1RAP	interleukin-1 receptor accessory protein	8.1	up	up	5.1
IL6	interleukin-6	8.0	up	up	28.2
IL6R	interleukin-6 receptor	2.8	up	up	2.8
IL10	interleukin-10	1.6	NA	up	10.7
IL12B	interleukin-12B	1.4	NA	up	2.7
IL23A	interleukin-23A	4.1	up	up	14.9
LIF	lymphocytes inhibitory factor	10.9	up	up	63.5
NEDD4L	ubiquitin-protein ligase NEDD4-like	4.9	up	up	7.2
OSM	oncostatin M receptor	1.4	NA	up	5.7
SOCS3	supressor of cytokine signaling 3	6.7	up	up	27.3
SOCS4	supressor of cytokine signaling 4	2.7	up	up	2.1
TGFBR1	tumor growth factor-β receptor 1	2.3	down	down	4.3
TNF	tumor necrosis factor	2.2	down	up	5.4
TNFAIP6	TNF-α interacting protein 6	3.1	up	up	6.0
TNIP3	TNFAIP3 interacting protein 3	1.8	NA	up	5.2
TNFSF8	TNF (ligand) superfamily, member 8	7.1	up	up	4.6
TNFSF9	TNF (ligand) superfamily, member 9	1.6	NA	up	3.8
TSLP	thymic stromal lymphopoietin	7.8	up	up	9.9
VEGFA	vascular endothelium growth factor A	3.9	up	up	6.9

Cytoskeleton/adhesion/migration

CTTN	contactin	3.2	up	up	7.5
COL8A2	collagen 8-α-2	24.0	down	up	2.1
DNM2	dynamin-2	19.8	up	up	16.5
EDN1	endothelin 1	1.1	NA	up	3.1
FILIP1	filippin-1	5.4	up	up	7.8
IQCG	IQ domain containing CG	10.6	up	up	5.6
IQGAP2	IQ domain containing GAP 2	11.6	up	up	4.2
MAP9	microtubules associated protein 9	3.4	down	up	2.7
TPM4	tropomyosin 4	1.7	NA	up	3.7
VIM	vimentin	3.6	up	up	11.5

Endocytosis/vesicular trafficking/phagocytosis

APBB2	amyloid beta precursor protein-binding, family B, member 2	16.0	down	up	2.1
SEP9	septin 9	13.1	down	up	3.4
STON2	stonin 2	2.8	up	up	11.8
VAMP2	vesicle-associated membrane protein 2 (synaptobrevin 2)	1.2	NA	up	2.3

Receptors/surface antigens

ANTXR1	antrax receptor 1	6.0	down	up	2.0
BAMBI	BMP and activin membrane-bound inhibitor	13.8	up	up	22.3
CD44	CD44 molecule	1.8	NA	up	2.2
ICHTHYIN	Ichtyin protein	20.4	up	up	88.6
TLR1	toll-like receptor 1	10.8	down	down	2.6

Signal transduction

AKAP11	protein kinase A anchoring protein A	2.8	down	down	7.4
TOLLIP	Toll interacting protein	2.5	up	up	2.8

Kinases/phosphatases/gene expression regulators

CREM	cAMP responsive element modulator	4.2	up	up	2.7
EGR1	early growth response 1	4.9	up	up	10.9
HIPK1	homeodomain interacting protein kinase 1	1.1	NA	up	2.1
JAK1	Janus kinase	1.6	NA	up	4.3
IRAK2	IL1 receptor-associated kinase 2	3.8	up	up	10.3
IRAK3	IL1 receptor-associated kinase 3	1.0	NA	up	2.2
IRF4	interferon regulatory factor 4	1.0	NA	up	2.8
IRF5	interferon regulatory factor 5	2.5	down	down	2.1
IRF6	interferon regulatory factor 6	1.6	NA	up	3.0
MAP2K3	MAP kinase kinase 3	6.2	up	up	7.5
MAP3K4	MAP kinase kinase 4	2.1	up	up	2.5
MAP3K8	MAP kinase kinase 8	1.4	NA	up	2.7
MAP3K7IP3	MAP kinase kinase kinase 7 interacting protein	2.5	up	up	3.4
MAPK8IP3	MAP kinase kinase kinase 8 interacting protein	7.4	down	up	3.3
MAPKAP1	Mitogen-activated protein kinase associated protein 1	3.5	down	down	2.2
MAPKAPK2	mitogen-activated protein kinase-activated protein kinase 2	1.0	NA	up	2.7
MKNK1	MAP kinase interacting serine/threonine kinase 1	4,7	down	down	4,9
SMAD6	SMAD family member 6	2,2	up	up	2,2
STAT5B	signal transducer and activator of transcription 5B	3,0	up	up	3,3
STK35	serine/threonine kinase 35	2,3	down	down	4,1
TNIP2	TNFAIP3 interacting protein 2	1,8	NA	up	2,6
TRAF1	TNF receptor-associated factor 1	6,3	up	up	13,9
TRAF3	TNF receptor-associated factor 3	2.8	down	down	4.4

TRIM25	tripartite motif-containing 25	2.9	up	up	6.6

Junction proteins

CLDN1	claudin 1	1.7	NA	up	2.1
CTNNB1	catenin beta-1	3.9	up	up	2.8
EMP1	epithelial membrane protein 1	2.3	up	up	2.2
PCDHB15	protocadherin beta 15	3.4	up	up	12.3
ZO-2	zona occludens 2	2.9	up	up	2.6

Apoptosis

BAG4	BCL2-associated athanogene 4	2.2	down	down	2.0
BCL2L1	BCL2-like 1	1.6	NA	up	2.3
BCL2L11	BCL2-like 11	2.1	down	down	2.9
BCL6	B-cell CLL/lymphoma 6	1.1	NA	up	2.4
BCL10	B-cell CLL/lymphoma 10	5.2	up	up	7.9
BCL11A	B-cell CLL/lymphoma 11A	2.4	up	up	3.7
BCOR	BCL6 co-repressor	3.3	down	down	4.6
CASP8	caspase 8	3.1	down	down	3.0
CASP12	caspase 12	5.0	up	up	5.4
CFLAR	CASP8 and FADD-like apoptosis regulator	2.3	up	up	2.0
CRADD	CASP2 and RIPK1 domain containing adaptor with death domain	1.4	NA	up	2.1
PERP	p53 apoptosis effector related to PMP22	1.8	NA	up	3.0
TRADD	TNFRSF1A-associated via death domain	2.2	down	down	2.0

Table 9. Gene expression in DC infected with EPEC or EcN. For convenience of analysis, genes were split into 5 groups, for each gene the symbol in the databases and its full name, fold of regulation to control (uninfected cells) for each strain following infection, and the direction of transcriptional alteration are shown. NA – not affected.

Gene Symbol	*Full name of the gene*	*Fold change EPEC*	*Regulation EPEC*	*Regulation EcN*	*Fold change EcN*

Immune response

CCL2	chemokine (C-C motif) ligand 2	8.6	up	up	19.1
CCL4	chemokine (C-C motif) ligand 4	179.9	up	up	370.3
CCL8	chemokine (C-C motif) ligand 8	5.4	up	up	8.0
CCL14/CCL15	chemokine (C-C motif) ligand 14/15	3.2	up	down	3.3

CCL18	chemokine (C-C motif) ligand 18	3.5	up	up	7.1
CCL20	chemokine (C-C motif) ligand 20	233.1	up	up	984.0
CCR1	chemokine (C-C motif) receptor 1	1.5	NA	down	2.2
CCR2	chemokine (C-C motif) receptor 2	5.8	down	down	4.8
CCR9	chemokine (C-C motif) receptor 9	4.6	up	up	2.3
CX3CR1	chemokine (C-X3-C motif) ligand 1	8.6	down	down	2.1
CXCL3	chemokine (C-X-C motif) ligand 3	121.0	up	up	766.8
CXCL1	chemokine (C-X-C motif) ligand 1	15.1	up	up	85.6
CXCL2	chemokine (C-X-C motif) ligand 2	31.9	up	up	166.1
CXCL6	chemokine (C-X-C motif) ligand 2	2.9	up	up	4.2
CXCL9	chemokine (C-X-C motif) ligand 9	10.7	up	up	14.9
CXCL10	chemokine (C-X-C motif) ligand 2	16.9	up	up	33.0
CXCL11	chemokine (C-X-C motif) ligand 11	5.4	up	up	13.3
CXCR7	chemokine (C-X-C motif) ligand 2	2.8	up	NA	1.3
IFNA7	interferon-α-7	2.2	up	NA	1.6
IFNA10	interferon-α-10	3.7	up	down	4.5
IFNB1	interferon-β-1	2.7	up	up	35.2
IL1A	interleukin-1A	7.3	up	up	21.6
IL1B	interleukin-1B	61.1	up	up	217.2
IL1RAP	interleukin-1 receptor accessory protein	4.2	up	up	5.2
IL6	interleukin-6	3.6	up	up	4.5
IL8	nterleukin-8	163.0	up	up	325.0
IL9R	interleukin-9 receptor	3.6	up	up	2.2
IL10RA	interleukin-10 receptor antagonist	2.1	up	NA	1.5
IL12A	interleukin-12A	2.6	down	down	5.0
IL12B	interleukin-12B	16.2	up	up	53.8
IL17C	interleukin-17C	2.3	up	NA	1.0
IL21R	interleukin-21 receptor	11.1	down	NA	1.4
IL23A	interleukin-23A	7.5	up	up	10.7
MIDN	midnolin	1.0	NA	down	15.0
OSTF1	osteoclast stimulating factor 1	1.5	NA	down	2.0
POSTN	periostin	1.9	NA	down	7.3
SOCS1	supressor of cytokine signaling 1	1.7	NA	down	2.2
SOCS3	supressor of cytokine signaling 3	28.2	up	up	68.8
SOCS4	supressor of cytokine signaling 4	1.5	NA	up	2.0
TNF	tumor necrosis factor	8.7	up	up	18.1
TNFAIP3	TNF-α interacting protein 3	15.3	up	up	32.3
TNFAIP6	TNF-α interacting protein 6	83.2	up	up	136.5
TNFSF9	TNF (ligand) superfamily, member 9	1.0	down	up	2.1
TNFSF12	TNF (ligand) superfamily, member 12	1.6	NA	down	2.2
TNFSF14	TNF (ligand) superfamily, member 14	2.3	down	down	5.1

Cytoskeleton/adhesion/migration

ADAM19	ADAM domain 19 (meltrin β)	4.4	up	NA	1.2
CNTN1	contactin-1	1.0	NA	up	13.1
CNTN3	contactin-3	1.1	NA	up	6.9
ITGA9	integrin-α-9	6.4	down	down	1.8
ITGA10	integrin-α-10	2.4	up	NA	1.4
ITGB3	integrin-β-3	2.2	up	up	6.6
MMP1	matrix metalloprotease 1	108.1	up	up	209.7
MMD2	matrix metalloprotease 2	1.4	NA	down	16.6
MMP12	matrix metalloprotease 12	8.1	up	NA	1.2
RASSF5	Ras association (RalGDS/AF-6) domain family member 5	1.7	NA	up	2.1

Endocytosis/vesicular trafficking/phagocytosis

CAV2	caveolin 2	2,0	down	NA	1,4
SEP9	septin 9	2,8	down	NA	1,1
STX4	syntaxin 4	1,9	NA	up	2,2
STX11	syntaxin 11	20,7	up	up	28,3
SYT5	synaptotagmin V	1,6	NA	up	10,7

Receptors/surface antigens

CD40	CD40 molecule. TNF receptor superfamily member 5	5.4	up	up	7.9
CD44	CD44 molecule	7.4	up	up	6.6
CD54 (ICAM1)	CD54 (intercellular adhesion molecule 1), human rhinovirus receptor	7.0	up	up	15.0
CD55	CD55 molecule, decay accelerating factor for complement (Cromer blood group)	1.9	NA	up	2.1
CD58	CD58 molecule	3.2	up	NA	1.5
CD69	CD69 molecule	4.6	up	up	11.1
CD80	CD80 molecule	11.9	up	up	9.7
CD83	CD83 molecule	17.6	up	up	24.4
CD109	CD109 molecule	2.0	up	NA	1.4
CD151	CD151 molecule	2.2	down	down	2.3
EGFR	epidermal growth factor receptor	2.5	up	NA	1.1
TLR1	toll-like receptor 1	6.0	down	NA	1.4
TLR3	toll-like receptor 3	10.7	down	down	5.1
TLR7	toll-like receptor 7	3.9	up	up	4.8
TRBC1	T cell receptor beta constant 1	2.2	up	NA	1.2

Signal transduction

CASS4	Cas scaffolding protein family member 4	1.0	NA	down	9.4
PALM2-AKAP2	paralemmin 2	2.0	down	NA	1.3
PALMD	palmdelphin	1.0	NA	down	9.8
RGS7	regulator of G-protein signaling 7	11.9	up	NA	1.0
TIRAP	toll-interleukin 1 receptor (TIR) domain containing adaptor protein	1.4	NA	down	2.1
WASF3	Wiskott-Aldrich syndrom factor-3	1.3	NA	down	8.3

Kinases/phosphatases/gene expression regulators

CNOT3	CCR4-NOT transcription complex, subunit 3	11.0	down	down	3.1
CPEB2	cytoplasmic polyadenylation element binding protein 3	4.3	up	up	2.2
DUSP4	dual specificity posphatase 4	9.9	up	up	4.2
EGR1	early growth response 1	8.6	up	up	17.8
EGR3	early growth response 3	40.9	up	up	70.8
IRAK2	IL1 receptor-associated kinase 2	17.2	up	up	16.3
IRAK3	IL1 receptor-associated kinase 3	1.3	NA	up	2.3
IRF4	interferon regulatory factor 4	1.7	NA	up	2.3
JAK3	Janus kinase 3	1.4	NA	down	3.3
MAPK7	Mitogen activated protein (MAP) kinase 7	1.8	NA	down	2.1
MAP2K2	MAP kinase kinase 2	1.7	NA	down	2.1
MAP2K6	MAP kinase kinase 6	3.1	up	up	9.9
MAP3K8	MAP kinase kinase kinase 8	5.5	up	up	8.5
MAP3K11	MAP kinase kinase kinase 11	1.5	NA	down	2.0
MAP3K12	MAP kinase kinase kinase 12	1.3	NA	down	3.5
MAP3K15	MAP kinase kinase kinase 15	1.5	NA	down	6.1
MAP3K7IP2	MAP kinase kinase kinase 7 interacting protein 2	1.9	NA	up	2.2
MAP3K7IP3	MAP kinase kinase kinase interacting protein 3	4.3	up	up	8.4
MAP4K1	MAP kinase kinase kinase kinase 1	3.4	down	NA	1.6
MAP4K4	MAP kinase kinase kinase kinase 4	2.4	up	NA	1.4
MAPK8IP3	MAP kinase 8 interacting protein 3	2.6	down	NA	1.3
MAPKAPK2	MAP kinase-activated protein kinase 2	1.5	NA	down	2.3
MARK2	microtubule affinity regulating kinase 2	5.5	down	NA	1.3
NFKBIA	nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, alpha	2.7	up	up	5.1
NFKBIB	nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, beta	4.0	up	up	16.5
NFKB1	nuclear factor of kappa light polypeptide	5.0	up	up	4.3

	gene enhancer in B-cells 1				
NKIRAS1	NFKB inhibitor interacting Ras-like 1	2.2	up	up	2.1
SMAD2	SMAD family member 2	2.3	down	up	8.1
SMAD4	SMAD family member 4	2.0	up	up	2.1
SMAD5	SMAD family member 5	2.0	down	NA	1.1
STAT4	signal transducer and activator of transcription 4	2.7	up	up	2.1
TGIF1	TGFB-induced factor homeobox 1	1.7	NA	down	3.4
TRAF1	TNF receptor-associated factor 1	6.1	up	up	4.1
TRAF3	TNF receptor-associated factor 3	2.3	up	NA	2.0
TRAF3IP3	TRAF3 interacting protein 3	13.1	down	NA	1.4
TRAF5	TNF receptor-associated factor 5	4.4	down	down	2.1
TNIP2	TNFAIP3 interacting protein 2	2.1	up	NA	1.3
TNIK	TRAF2 and NCK interacting kinase	1.8	NA	down	10.9

Junction proteins

CDH8	cadherin 8 type 2	4.4	up	up	4.0
CDH10	cadherin 10 type 2	2.2	down	NA	1.4
CDH26	cadherin-like 26	1.5	NA	up	2.3
CLDND1	claudin domain containing 1	1.3	NA	up	2.1
CLDN11	claudin 11	1.2	NA	down	5.0
CLDN18	claudin 18	2.4	up	NA	1.1
CLDN19	claudin 19	1.8	NA	up	2.1
CLDN23	claudin 23	2.9	down	down	3.0
GJA5	gap junction protein alpha 5	2.8	down	NA	1.2
GJC1	gap junction protein gamma 1	6.5	down	NA	1.3
PCDHA1	protocadherin alpha subfamily C 1	5.1	down	down	2.1
PCDH7	protocadherin 7	2.8	down	down	2.6
PCDH12	protocadherin 12	2.2	down	down	1.3

Apoptosis

BAD	BCL2-antagonist of cell death	5.2	down	NA	1.2
BAG4	BCL2-associated athanogene 4	3.4	down	NA	1.7
BCL2A1	BCL2 associated protein 1	13.4	up	up	19.0
BCL2L1	BCL2-like 1	1.5	NA	down	5.1
BCL2L11	BCL2-like 11	2.7	up	up	4.2
BCL6	B-cell CLL/lymphoma 6	16.4	up	up	23.8
BCL11B	B-cell CLL/lymphoma 11B	1.1	NA	down	3.7
BOK	BCL2-related ovarian killer	1.4	NA	down	4.2
CASP2	caspase 2	2.1	down	down	2.3
CASP3	caspase 3	1.4	NA	up	2.1

CASP12	caspase 12	7.5	up	up	7.3
CFLAR	CASP8 and FADD-like apoptosis regulator	2.5	up	up	4.0
CUL1	cullin 1	1.0	NA	up	2.2
CUL3	cullin 3	6.6	up	up	13.0
NDFIP2	Nedd4 family interacting protein 2	2.5	up	NA	1.0
P53AIP1	p53-regulated apoptosis-inducing protein 1	4.2	down	NA	1.2
THAP3	THAP domain containing, apoptosis associated protein 3	4.4	down	NA	1.2

Table 10. Gene expression in THP-1 infected with EPEC or EcN. For convenience of analysis genes were split into 5 groups. For each gene the symbol in the databases, its full name, fold of transcriptional changes vs. control (uninfected cells) for each strain following infection, and the direction of alterations are shown.

The expression of genes of interest that were identified in both arrays was compared between THP-1 cells and DC. Table 10 indicates gene expression changes in infection with EPEC or EcN relative to non-infected control cells. Interestingly, IL-1 and neutrophil chemoattractants are expressed in much higher extent in THP-1 cells than in DC. In infections with EcN these cytokines were up-regulated to a higher extent in both cell types than in EPEC infections. As pathogenic organisms have developed mechanisms to subvert or modulate the immune system in one way or other, these findings might indicate a specific influence of EPEC bacteria on cytokine expression. An influence of EPEC on cytokine signaling has been demonstrated in several studies and it had been observed that EPEC infections do not cause severe inflammation (Ashkenazi *et al.*, 1983; Nataro and Kaper, 1998).

IL-6, however, was up-regulated to a much higher extent in DC and also EcN infection enhanced expression of IL-6 in higher extent than EPEC infection. IL-6 was recently shown to be involved in anti-inflammatory processes in DC by blocking NF-κB binding activity (Hegde *et al.*, 2004).

IL-23 was also expressed to a larger extent in infections with EcN and in DC even higher than in THP-1 cells. In contrast, in infections with EPEC THP-1 cells up-regulated IL-23 to a higher extent than DC.

Cell type	*Gene symbol*	*EPEC*		*EcN*	
		fold change	regulation	fold change	regulation

Immune response

THP-1	**CCL2**	8.6	up	19.1	up
DC		3.3	up	8.2	up
THP-1	**CCL14 /// CCL15**	3.2	up	3.3	down
DC		1.8	NA	2.2	up
THP-1	**CCL20**	233.1	up	984.0	up
DC		688.8	up	1882.2	up
THP-1	**CXCL1**	15.1	up	85.6	up
DC		1.2	NA	4.1	up
THP-1	**CXCL2**	31.9	up	166.1	up
DC		3.4	down	10.7	up
THP-1	**CXCL3**	121.0	up	766.8	up
DC		1.5	NA	6.8	up
THP-1	**CXCL11**	5.3	up	8.5	up
DC		33.7	up	15.1	up
THP-1	**IL1A**	7.3	up	21.6	up
DC		1.9	NA	13.2	up
THP-1	**IL1B**	61.1	up	217.2	up
DC		3.8	up	6.1	up
THP-1	**IL1RAP**	4.2	up	5.2	up
DC		5.2	up	4.2	up
THP-1	**IL6**	3.6	up	4.5	up
DC		8.0	up	28.2	up
THP-1	**IL12B**	16.2	up	53.8	up
DC		1.4	NA	2.7	up
THP-1	**IL23A**	7.5	up	10.7	up
DC		4.1	up	14.9	up
THP-1	**SOCS3**	28.2	up	68.9	up
DC		6.7	up	27.3	up
THP-1	**SOCS4**	1.5	NA	2.0	up
DC		2.7	up	2.1	up
THP-1	**TLR1**	6.0	down	1.4	NA
DC		10.8	down	2.6	down
THP-1	**TNF**	8.7	up	18.1	up
DC		2.2	down	5.4	up
THP-1	**TNFAIP3**	12.9	up	37.3	up
DC		2.1	up	9.8	up

THP-1	**TNFAIP6**	83.9	up	123.3	up
DC		3.1	up	6.0	up
THP-1	**TNFSF9**	1.0	NA	2.1	up
DC		1.6	NA	3.8	up
THP-1	**TNFSF14**	2.3	down	5.1	down
DC		129.2	up	116.5	up
THP-1	**TSLP**	1.4	NA	3.9	up
DC		9.9	up	7.8	up

Endocytosis/vesicular trafficking/phagocytosis

THP-1	**SEP9**	2.8	down	1.0	NA
DC		3.4	up	13.1	down

Receptors/surface antigens

THP-1	**CD44**	7.4	up	6.6	up
DC		1.8	NA	2.2	up

Kinases/phosphatases/gene expression regulators

THP-1	**EGR1**	8.6	up	17.8	up
DC		4.9	up	10.9	up
THP-1	**IRAK2**	18.2	up	39.3	up
DC		3.8	up	10.3	up
THP-1	**IRAK3**	1.3	NA	2.3	up
DC		1.0	NA	2.2	up
THP-1	**IRF4**	1.7	NA	2.3	up
DC		1.0	NA	2.8	up
THP-1	**MAP2K3**	2.9	up	2.2	up
DC		6.2	up	7.5	up
THP-1	**MAP3K7IP3**	4.3	up	8.4	up
DC		2.5	up	3.4	up
THP-1	**MAP3K8**	13.8	up	23.6	up
DC		1.4	NA	2.7	up
THP-1	**MAPK8IP3**	2.6	down	1.3	NA
DC		7.4	down	3.3	up
THP-1	**MAPKAPK2**	1.5	NA	2.3	down
DC		1.0	NA	2.7	up
THP-1	**TRAF1**	6.0	up	4.1	up
DC		6.3	up	13.9	up

Apoptosis

THP-1	**BAG4**	2.9	down	1.6	NA
DC		2.2	down	2.0	down
THP-1	**BCL2L11**	2.5	up	1.5	NA
DC		2.0	down	2.9	down
THP-1	**BCL6**	16.4	up	23.8	up
DC		1.0	NA	2.4	up
THP-1	**CASP12**	7.5	up	7.3	up
DC		5.4	up	5.0	up
THP-1	**CFLAR**	2.2	up	2.4	up
DC		2.3	up	2.0	up

Table 10. Comparison of gene expression between THP-1 and DC infected with EPEC and EcN.

3.8. Analysis of cytokine expression by DC following infection with EPEC, EcN, and C600 employing a cytokine array

To assess cytokine expression levels for a set of pro- and anti-inflammatory cytokines as a measure for possible additional immunomodulatory effects of EPEC a Cytokine Array (RayBio) was used. For this, freshly differentiated human DC were infected with EPEC, EcN, or C600 for 2 hours, and then gentamycin to a final concentration of 100 µg/ml was added with 1 ml of fresh medium for 16 additional hours. The supernatants were collected and the presence of cytokines in the supernatant was determined as described in Materials and Methods. The results obtained after subtracting background values are summarized in Fig. 24 in comparison with values obtained from control uninfected cells.

As could be expected, IL-10 expression was found to be much higher for non-pathogenic *E. coli* strains. As was already apparent from data obtained by DNA microarray the transcription of pro-inflammatory cytokines was enhanced in cells infected with C600 and EcN compared to EPEC infected cells. For IL-12 p70 the highest expression values were found with cells infected with C600. Expression of IL-12 p70 following EcN and EPEC infection was low. IL-12 p40 was expressed to a much higher extent in EcN and C600 infected cells than in EPEC infected cells. However, it has been shown in several studies that the p40 subunit in its

dimeric form is able to bind to the IL-12 receptor. In this way the functional heterodimeric IL-12 p70 is blocked from receptor-binding so that no signaling functions via the IL-12 receptor can be transmitted (Ling *et al.*, 1995; Gately *et al.*, 1998).

Accumulating evidence provides further support for on the immunosuppressive capacity of EPEC as this was also demonstrated for epithelial cells and IL-8 secretion using the Caco-2 cell line (Ruchaud-Sparagano *et al.*, 2007). This is further supported by IL-8 expression in DC that was found to be higher for EcN and C600 infected cells than for EPEC infected (where it was comparable to control untreated cells).

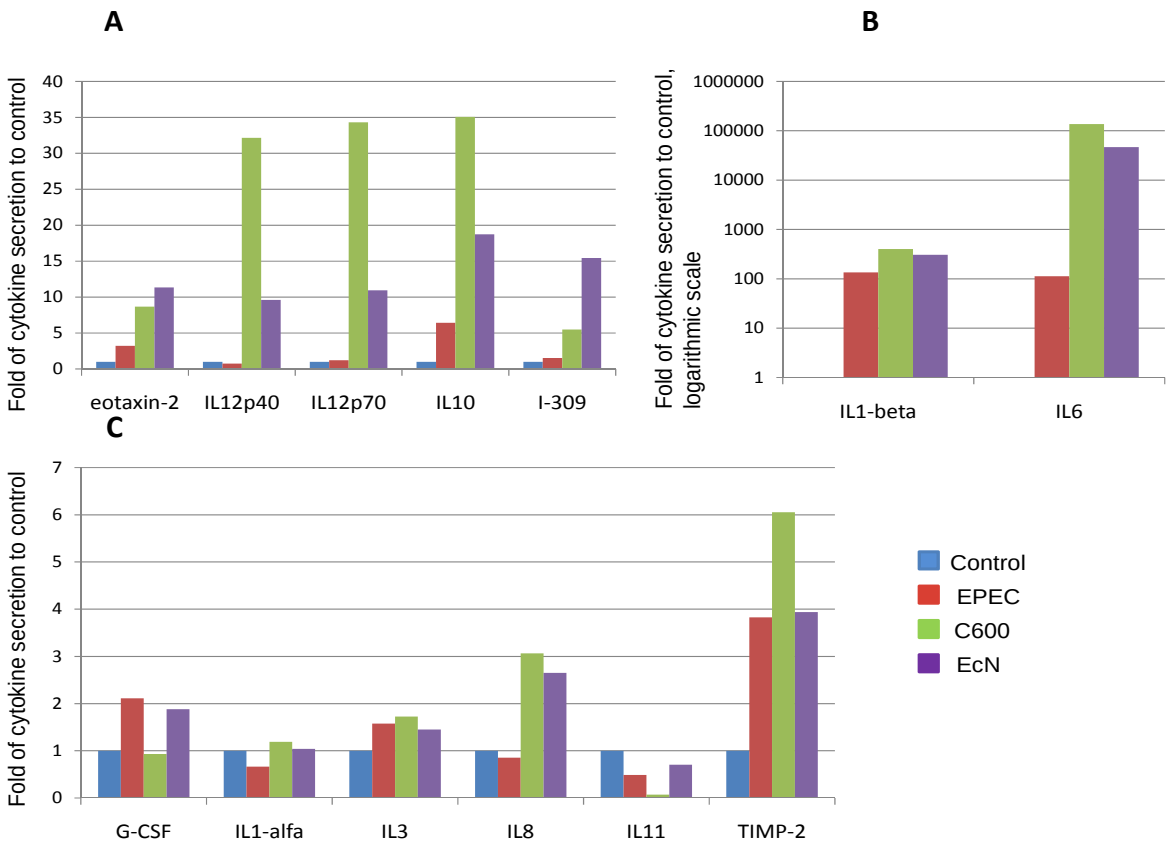


Fig. 24. Bars reflect the ratio of chemilumeniscence intensities of non-infected control cells vs cells infected with the indicated strains. For these experiments 1 ml of supernatant from DC that were previously infected with the indicated strains was used (MOI of 10, infection time 2 hours, expression time 16 hours). **A**) ratios from 10 to 35; **B**) ratios more than 35, logarithmic scale; **C**) ratios less than 10. The experiment was performed with 2 spots with antibodies on the membrane specific for each cytokine.

3.9. Expression of IL-10 and IL-12 after infection with EcN and EPEC

Following infection of human DC with EcN and EPEC the expression of IL-10 and IL-12 was assessed as these are among the most important anti- and proinflammatory cytokines. The results are represented in Fig. 25.

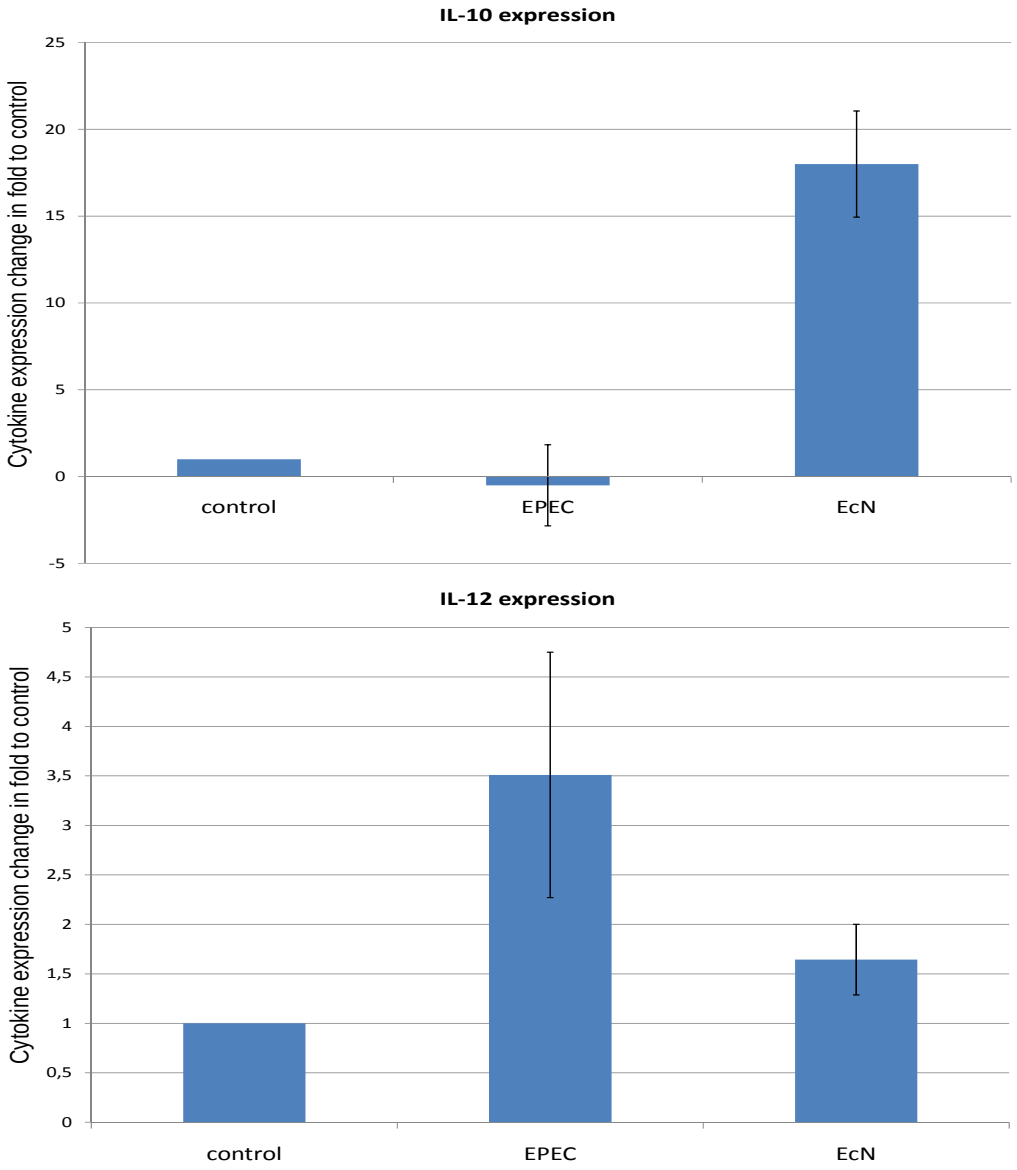


Fig. 25. IL-10 and IL-12 expression in DC infected with EPEC or EcN in fold to control as assessed by qRT-PCR. Error bars reflect standard error. The experiments were performed 3 times in triplicate. The error bars represent the standad error.

In accordance to the microarray data obtained for cells infected with EcN, IL-10 expression was much higher than in EPEC-infected cells. IL-10 expression also exceeded IL-12 expression by both EcN and EPEC infected cells. For the IL-12 cytokine the p35 subunit was determined and the results differed from previous data obtained from analysis using microarrays, where only the expression data for IL-12p40 passed validation. In the microarray analysis the IL-12 p40 expression in EcN infected cells was approximately double of expression in EPEC infected cells, while in qRT-PCR p35 expression was approximately 2-fold higher in EPEC treated cells than in EcN infected cells. As many pathogenic bacteria induce a way more profound up-regulation of IL-12p35 the results obtained for the prototype EPEC strain E2348/69 can be considered as a further indication of the immunomodulatory effect of EPEC.

3.10. Analysis of micro-RNA profiles array in infected THP-1 cells

There is growing evidence that microRNAs (miRNAs) play a role in the regulation of various cellular processes. Most of the mRNAs in the cell have indeed numerous sites where miRNAs can bind and in this way not only prevent translation but also accelerate mRNA degradation. Many recent studies already showed the involvement of certain miRNAs, such as miR-155 (Rodriguez *et al*., 2007) and miR-146 (Taganov *et al*., 2006) in the regulation of immune responses. To analyze the differential expression of miRNAs in monocytes infected with different *E. coli* strains a miRNA array analysis was performed. THP-1 cells were treated in the same way as for gene expression analysis using microarrays. All cellular RNAs were prepared using the TRI reagent for preservation. Further analysis and array hybridization was performed at the Integrated Functional Genomics (IFG, Medical Faculty, University of Münster). For that isolated miRNAs were labeled and hybridized with the array (Febit) containing complementary oligonucleotides.

A list of affected miRNA with P-values of less than 0.05 is presented in Table 12.

miRNA name	Fold C600	Fold EPEC	Fold EcN	Short names of a target genes and quantity of binding sites
MIR-137	20.4	NA	3.0	3: PALM2-AKAP2; AKAP2; NFIB; COLLAGEN 19 ALPHA 1; 2: DUSP4; CD83; MMP20; MAP4K5; COLLAGEN 11 ALPHA 1; CENTAURIN BETA 2; BCL2; RAB1A.
MIR-21	8.2	2.5	2.5	3: MYOSIN6; RAB22A; CDK6; MMP16; 2: MAP3K7IP3; ERMIN; INTEGRIN BETA3; SOCS5; CREB5; RAB11A; SMAD5;
MIR-23A	NA	NA	2.1	6: CARD8; 3: SMAD5; NFIB; XIAP; MAPK1IP1L; SYT4; MUC21; 2: MAP3K7IP3; CXCL12; STX6; ADAM12; NRXN3; BCL2; MAP4K4; MAP7; VAMP4; MAP3K3; VEGFA; STX17;
LET-7F	5.3	NA	2.4	3: MAP3K1; 2: PAK1; MIRNA 155; HLA-DQA1; ADAM15; CCL22; MYO5B;
MIR-20A	NA	NA	2.2	5: ITGB8; BCL11B; 3: NEDD4L; NFIB; SMAD1; SOCS4; CADM2; 2: PCDHA12; PCDHA13; PCDHAC1; PCDHAC2; ATXN1; RAB22A; CNOT6; STAT3; IL17RD; CREB5; ADAM12; TFAP4; CBEP1; DYNC1LI2; SMAD5; TNFAIP8; COL4A1; CD47; MAP3K7IP3; MARK1; IL28RA; RABGEF1;
MIR-548A-3P	28.6	NA	3.1	5: CREB5; CALM1; 4: SOCS6; PIK3R1; CD47; 3: XIAP; CLDN12; RAB22A; SMAD5; NFAT5; MMD; SOCS4; MYO5D; 2: RABL3; LEPR; CXCR4; AKAP6; PLCB1; DYNC1LI2; JAK2; CXCR7; PLCH1; IL1RAP; RAP2A; CPEB2; TGFBR1; RAB18; MAP7; SMAD4; VEGFA; TLR4; RAB8B; SNX11; RALBP1; RBM12; ATP2A2; MAP3K2; NFIB; CCNJ; MARK1; IL1A;
MIR-553	10.9	2.9	2.8	2: CD47; STXBP5; PCDH19; RAB12;
MIR-580	39.7	2.0	3.7	4: ADAMTS5; 3: COLL11A1; MAP3K1; XIAP; miRNA155; AKAP2; HIPK1; MMP16; 2: TGFBR1; COL4A4; CDH26; NFAT5; SNX13; MAP4; IL1A; IL13RA1; SEPT6; ITGA1; SEP15; STX7; VCL; SEPT10; HIF1A; PRKAR1A; IL1RAP; CPEB3; CARD8; CD200R1; CPEB4; SNX9; PALM2; TNFAIP3; SOCS6; PCDH11Y; PCDH11X;
MIR-633	59.5	2.1	4.8	3: CXCL5; NFAT5; MAP9; CPEB2; SOCS6; 2: MUC13; NFIB; MMP16; CD164; PCDH18; EBF3; IL1RAP; LAMP2; SNX16; BCL11A; PCDH19; MACF1; AKAP6; RAB11A; PCDH11X; FBN1; COL5A2; SOCS5;
MIR-651	7.7	NA	2.8	3: MAP3K2; 2: BCL2; MAP2K4; PAK7; LAMP2; MAP3K7IP3; MAP4K5; VEGFA; ITGB8; ITGAV; IRF2;
MIR-9	38.7	NA	3.6	4: CNOT6L; FBN1; NFIB; CTNND1; 3: JAK1; PCDH11X; ITGA6; BCL2; CENTD3; 2: CPEB3; miRNA155; ADAMTS5; CPEB2; RIPK5; SOCS5; N4BP3; COL12A1; CLDN18; MAP3K7IP3; TPM3; ANTXR1; MAP7; DYNC1LI2; CPEB4; DUSP10; MMP16; TNFAIP8; CARD10;

				IL16; CCL4L1; STXBP5; RAB21; HIF3A; SOCS2; COL4A4;
MIR-98	29.7	2.2	4.0	3: miRNA155; PAK1; SYT9; 2: MAP3K1; PCDH19; CNTN3; SOCS7; ADAM15; AKAP6; AKAP11; CREB5; MAP4K4; IL17RD;
MIR-542-3P	48.1	2.0	4.7	3: MAP1B; miRNA155; AKAP11; 2: SYT4; IL33; PALM2-AKAP2; AKAP2; MYO3B; IL1RAP; MAP2K4; MAPK1IP1L; MMP24; SNX16; CCL22; STX12; COL4A3;
MIR-338-3P	30.4	NA	2.5	3: AKAP1; NFIB; IL17RD; 2: RAB14; ITGA5; ANXA2P3; MAP3K3; IL11RA; TPM1; TNFSF11; PCDH19; MYO3B; VAMP4;
MIR-29B	21.1	NA	2.9	2: COL5A1; COL11A1; TPM1; PCDH17; PCDH19; COL4A3; AKAP11; RAB15; DYNC1I1.
MIR-376A	16.9	2.1	3.5	2: CNOT6; NFIB; MAP4K3; CD200; CTNNBL1; SNX13; PCDH11X; ITGA6; NFAT5; RAB10;
MIR-1	37.2	2.1	3.5	3: BCL2L2; 2: MAP3K1; IL33; VAMP2; COL6A3; RGS7; BCL11A; CBEP4; HIPK1; ADAMTS3; RAB6A; SMAD5; IL1RAPL1; RAB6C; BCL2; IL22;
MIR-32	84.0	NA	3.6	4: SYT4; 3: CD69; CPEB2; HIPK1; 2: RAB14; CPEB4; BCL11B; BCL2L11; BCL11A; ADAM10; PCDH11X; SOCS5; GJC1; MAP2K4; PCDH17; IQGAP2; BCL2; CD59; EGR3;
MIR-513A-3P	21.2	3.9	2.7	5: INTEGRIN BETA8; 3: MMP16; SYNTAXIN BINDING PROTEIN 4; PRKA CB; CAVEOLIN1; MYOSIN 6; SMAD5; 2: VAMP4; CULLIN5; NEXIN; SORTING NEXIN4; SMAD2; CULLIN3; CD44; CENTAURIN ALPHA2; HA-DQ ALPHA1; PRKA G2; BCL2; CASP10; RAB18;
MIR-519C-3P	55.0	2.6	2.7	4: COL4A4; AKAP11; 3: BCL2L2; CNOT6L; BCL11A; SMAD5; MAP2.
MIR-937	16.3	4.3	3.2	2: IKBKG; ARFGAP1.
MIR-935	9.3	3.6	3.2	3: CREB5; 2: CNOT7; IL11RA;
MIR-106A	6.0	2.5	1.7	5: BCL11B; 3: PCDHA1 - 13; ITGB8; NEDD4L; TNFAIP1; BCL2L2; SOCS4; NFIB; RGS17; COL4A1; 2: MAP3K2; COL19A1; RAB5B; STAT3; CASP7; RAB18; RIPK5; SNX11; MAPK1IP1L; SOCS6; SMAD1; CREB5; IL28RA; IL17RD; PCDH20; ADAM12; MAPK14; CADM2; MAP3K5; TNFAIP8; HIPK1; RAB10; TGFBR2;
MIR-513B	35.1	NA	3.3	3: IRF2; PCDH17; RASSF2; 2: PALM2; CUL4A; SMAD2; MARK1; CXCL12.

Table 12. miRNAs expression result for THP-1 infected with EPEC, C600 or EcN. Columns show fold of regulation to control uninfected cells. Gene's short name in databases is indicated. The number of binding sites is indicated before the list of genes.

3.11. Establishing a Caco-2 co-culture with human DC employing a Transwell filter system

As the functionality of the gastrointestinal barrier requires constant cross-talk between immune cells and epithelial cells, it is important to use relevant model systems to study host-pathogen interactions in the gut. To this end a tissue culture model based on the cultivation of the Caco-2 cell line on Transwell filters with DC was established in the laboratory (Rescigno *et al*., 2001). In this set-up the epithelial cells were grown for 2 weeks in a way that their basolateral side was facing the upper compartment of the Transwell filter system (see Materials and Methods).

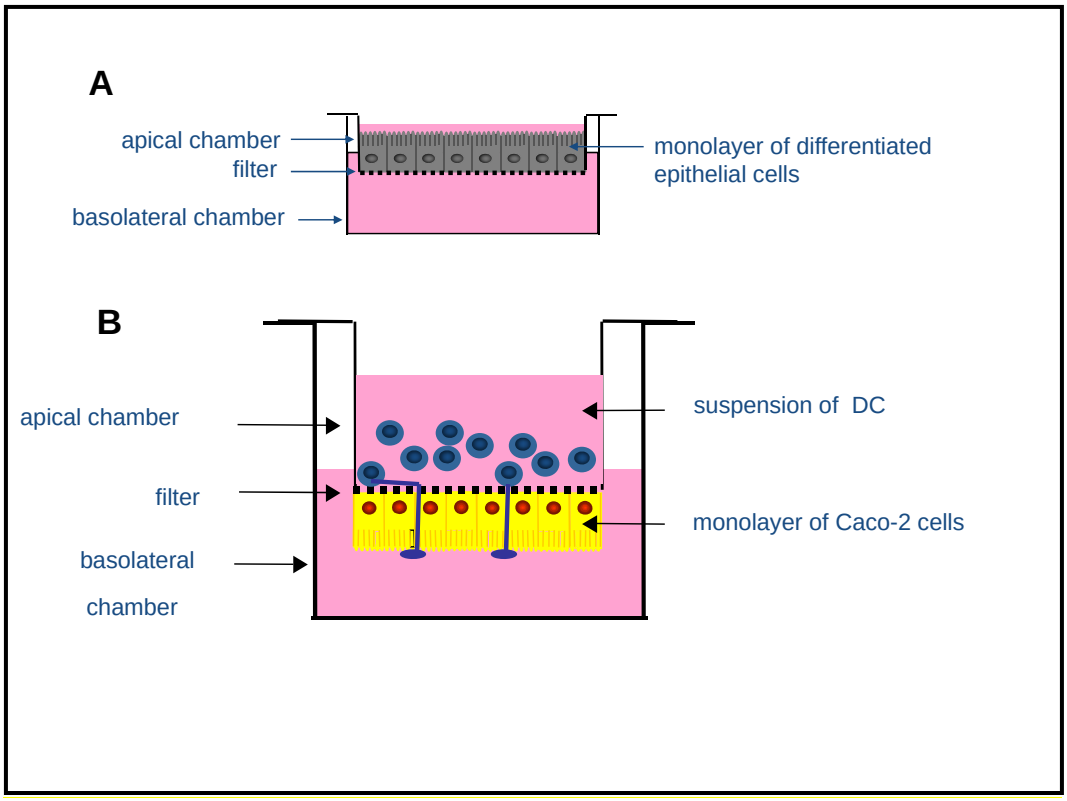


Fig. 26. Scheme of Transwell filter based coculture system of Caco-2 cells and human DC. A – Caco-2 cells grown in 'normal' orientation on Transwell filter; B – set-up used in this study, when Caco-2 are grown upside down and DC are added to upper compartment (hence to basolateral side of epithelial layer) of the Transwell.

DC were added to the upper compartment and bacteria to the lower compartment. This tissue culture system allows the epithelial cells to polarize. Hence the bacteria in the lower compartment only interact with the apical surface of the cell layer and the DC are added to the basolateral side and can respond to signals from the epithelium. In this set-up DC are also able to reach the apical surface with their protrusions as exemplified in Fig. 26 and possibly also sample bacteria.

In these experiments DC were incubated with EPEC and EcN bacteria for 2 h of infection with a MOI of 50. Fig. 27 depicts DC labeled with the active-ester dye CFSE. The images were obtained with confocal laser scanning microscopy and the specific Z-stacks were then used to create a depth-coded view of the selected area. Blue colors represent Z-levels on the basolateral side of the epithelial layer (upper compartment of the filter), green colors represent the level of the membrane filter pores, yellow and red indicate the internal parts of the Caco-2 layer or the apical side of the polarized epithelial cells.

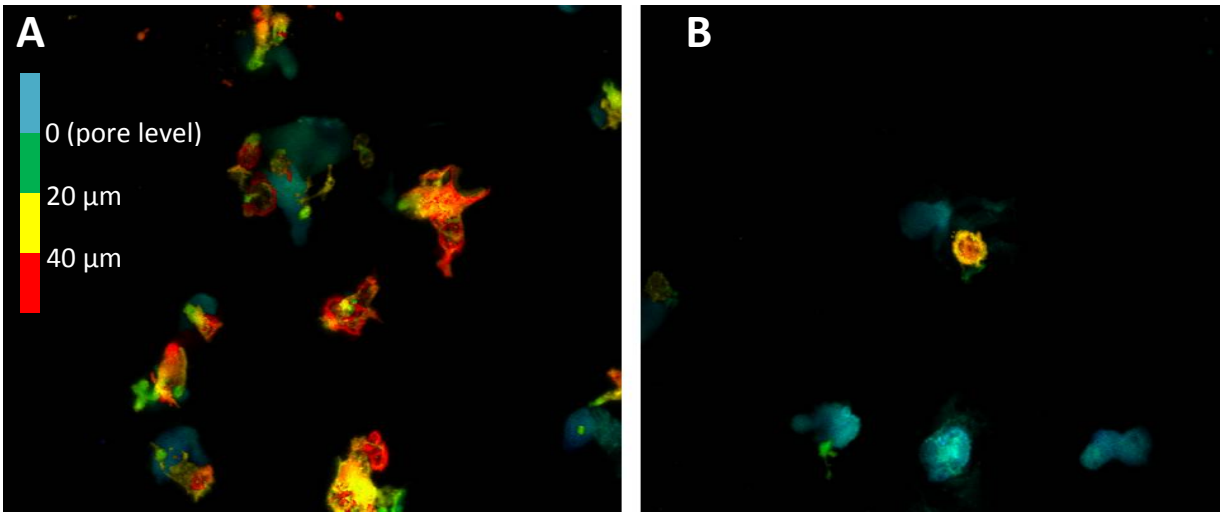
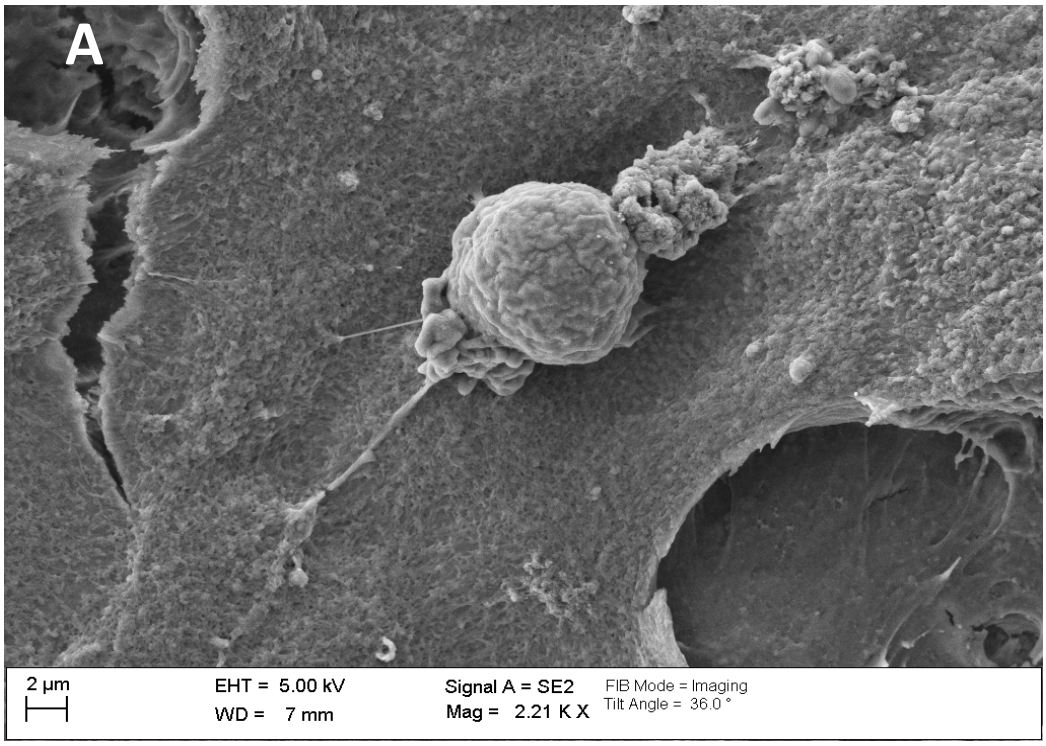
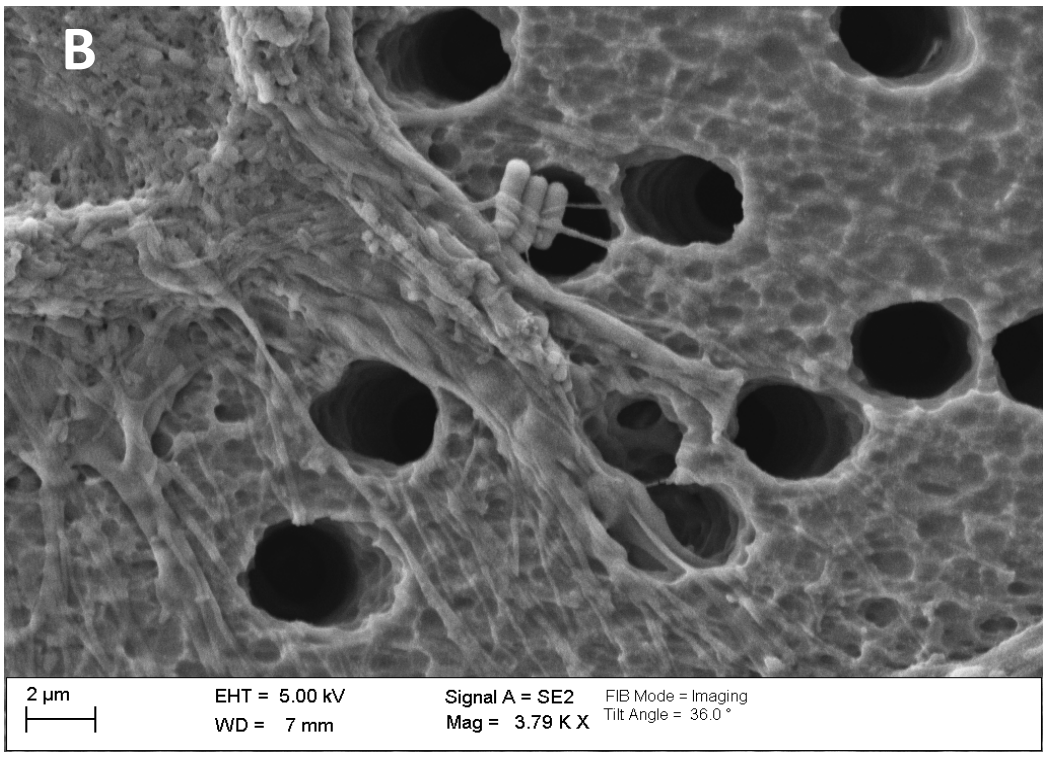
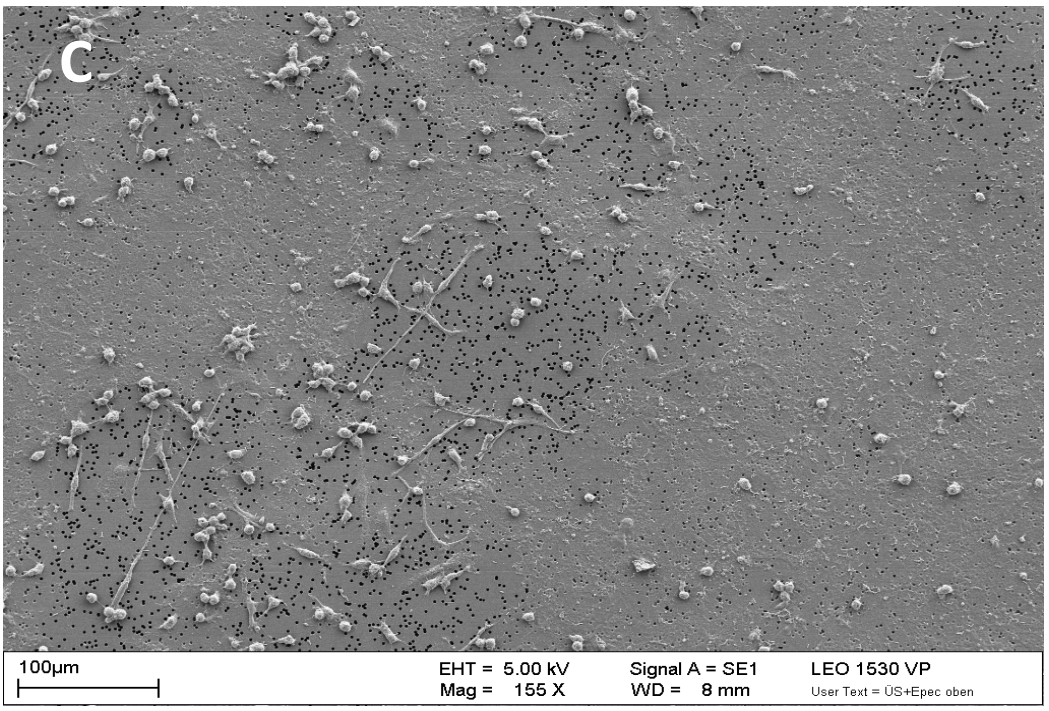


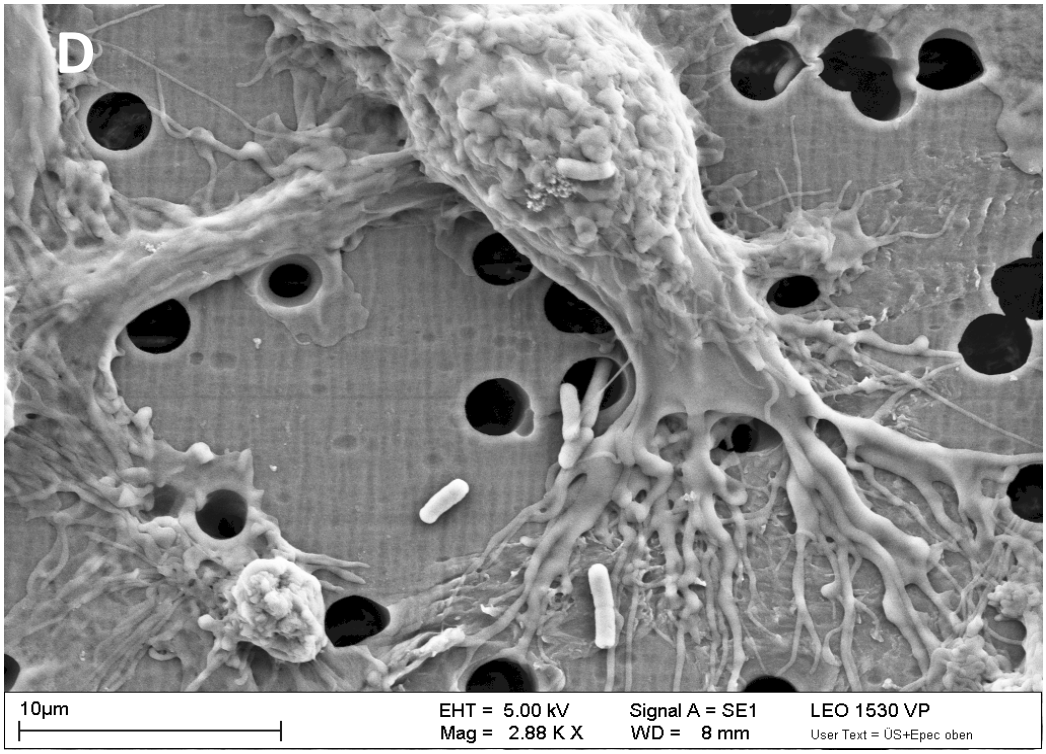
Fig. 27. Depth code applied to Z-stack acquired on confocal microscope. Blue are the cells at the basolateral side of the IECs, green is level of the pores, yellow and red are protrusions from pores to apical side of the IECs (changing from yellow to red is moving from the pores to apical side). (**A**) – filter infected with EcN; (**B**) – filter infected with EPEC.

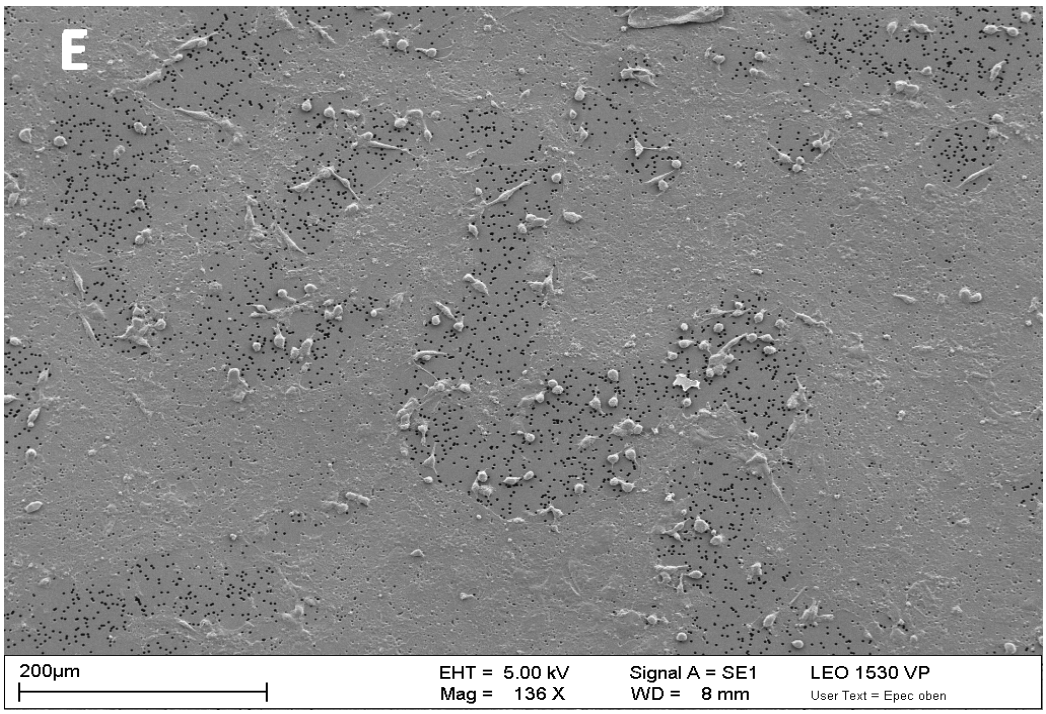
To further investigate differences in responses to infection with EPEC and EcN in this co-culture system of Caco-2 and DC scanning electron microscopy (SEM) was applied. For this the same infection conditions as previously used for immunofluorescence (2 hours infection with EPEC or EcN in MOI of 50) were employed. After preparation for SEM (see Materials and Methods) the filters were scanned from the apical side of the epithelial layer. For EPEC no protrusions to the apical side were detected, for EcN one balloon-shaped protrusion edge and one flatly spread protrusion in close contact with bacteria could be identified (Fig. 28, A and B). In subsequent experiments we only infected filters with EPEC. To check if EcN supernatant have some effect on formation of protrusions or uptake of bacteria by DC to one half of the filters 10 µl of EcN supernatant was added to the upper compartment of the filter simultaneously to the infection with EPEC. In this case the filters were scanned from both the apical and the basolateral side of the epithelial layer. On the view from the basolateral side it was noticed that in filters treated with EcN supernatants DC were prone to form longer dendrites (Fig. 28 C, D against E, F). From the apical side no protrusions were seen in any of the filters analyzed.




B
2 µm
EHT = 5.00 kV
WD = 7 mm
Signal A = SE2
Mag = 3.79 K X
FIB Mode = Imaging
Tilt Angle = 36.0 °


C
100µm
EHT = 5.00 kV
Mag = 155 X
Signal A = SE1
WD = 8 mm
LEO 1530 VP
User Text = US+Epec oben


D
10µm
EHT = 5.00 kV
Mag = 2.88 K X
Signal A = SE1
WD = 8 mm
LEO 1530 VP
User Text = ÜS+Epec oben


E
200µm
EHT = 5.00 kV
Mag = 136 X
Signal A = SE1
WD = 8 mm
LEO 1530 VP
User Text = Epec oben

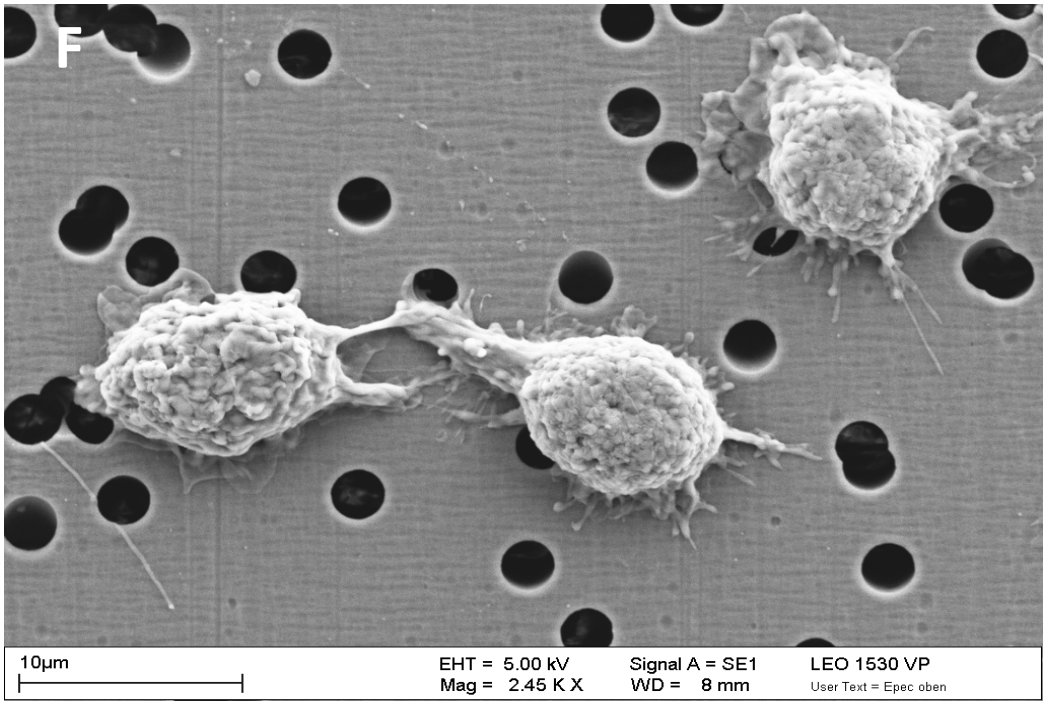


Fig. 28. Co-culture Transwell filter system with Caco-2 and DC infected with EcN from the apical side and with EcN supernatant added from the basolateral side, view from the apical side of epithelial layer **(A, B)**, edge of the DC protrusion **(A)** and bacteria that are being taken up by DC **(B)** can be seen. **(C, D)**: filter infected with EPEC with EcN supernatant present, view from the basolateral side. **(E, F)**: filter infected with EPEC without EcN supernatant, view from the basolateral side.

3.12. DC are susceptible to EPEC antiphagocytotic effects and show only slight differences in uptake rates between C600 and EcN

To assess the efficiency of phagocytosis of human DC these were infected with GFP-overexpressing bacteria (EPEC, EcN, and C600) for 1 hour. Subsequently, the cells were washed and fixed, and subjected to flow cytometric analysis. For flow cytometric quantification of invasion the method described by Pils *et al.* for epithelial cells was used with slight modifications to allow to quantifiation also for non-adherent cells (Pils *et al*., 2005). The

uptake index was calculated as the number of GFP-positive cells multiplied by mean fluorescence.

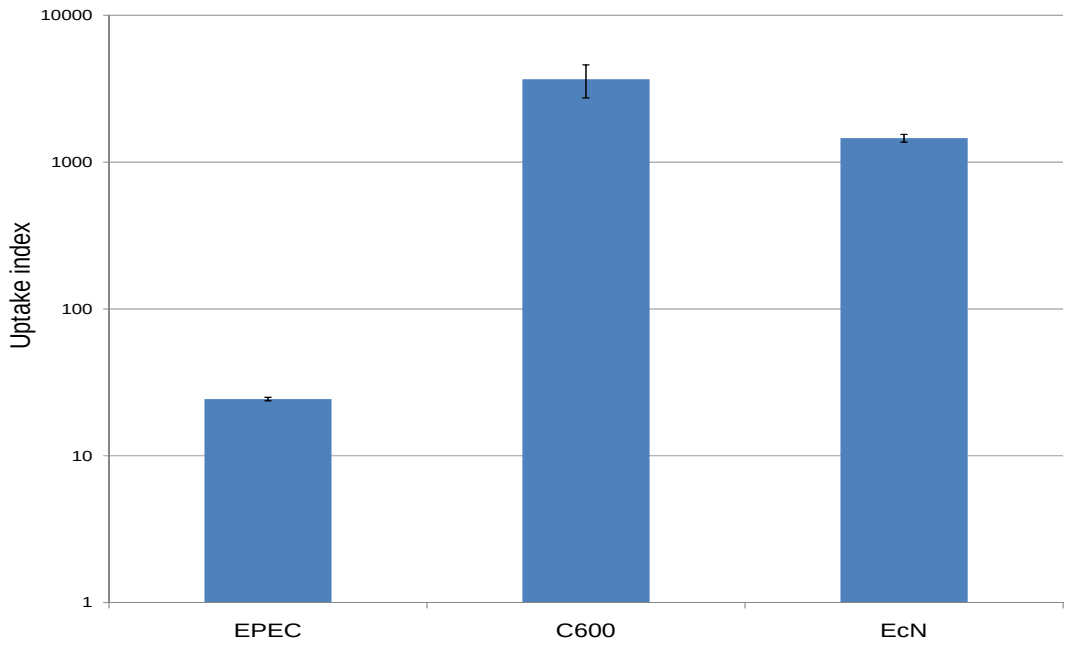


Fig. 29. Phagocytosis of different *E. coli* strains by DC. The bars reflect the uptake index obtained for each bacterial strain (logarithmic scale). Uptake index was calculated as percentage of GFP-positive cells multiplied by mean fluorescence intensity (Pils *et al.*, 2006). The experiments were performed 4 times in triplicate. The error bars represent the standard error.

The phagocytosis of EPEC bacteria by human DC is significantly reduced (Fig. 29) which can be observed already after 1 hour. Indeed, the uptake index values for EPEC never exceeded 50, while for C600 and EcN approached 8000.

4. Discussion

4.1. EcN microcins exert a differential effect on diarrhoeagenic *E. coli* strains

The number of bacterial cells in the human body exceeds the number of eukaryotic cells by a factor of 10 (Backhed *et al*., 2005). For intestinal pathogens this implies that the very first barrier they will meet in the gastrointestinal tract are other bacteria. Therefore, strategies for outcompeting members of the normal gut microbiota are very important. Many intestinal bacteria, however, possess specific mechanisms to prevent pathogens (or other microbes) from destroying or occupying their particular niches. Some of these bacteria have been identified long ago and are known as probiotics. Most, however, still remain uncharacterized due to difficulties in cultivation. The importance of normal gut microbiota that evolved in long coevolution processes with mankind is easy to observe in patients treated with antibiotics. These patients have a much higher rate of infections with enteropathogenic bacteria that are able to colonize the gut when the additional barrier and competition due to the presence of commensals has been destroyed.

Some strains of *E. coli* are producing bactericidal or bacteriostatic compounds called microcins, which affect mostly closely related bacteria (within the species or the genus and sometimes also act on members of the same family) (Duquesne *et al*., 2007). *E. coli* Nissle 1917 (EcN) discovered almost a century ago in 1917 (Nissle, 1925) has also been shown to secrete microcins (Patzer *et al*., 2003). As was shown in this study, many diarrhoeagenic *E. coli* strains are affected by EcN microcins. This might explain at least part of the effects reported for EcN against pathogens and might be further exploited as a basis for the development as a diarrhoea cure alternative to antibiotics as these are also harmful for normal microbiota. Although up to know it has been impossible to obtain active preparations of EcN microcins, it has certainly been possible to use live EcN bacteria as therapeutics given the fact that Mutaflor has been used for decades and thus far has not been associated with

any side-effects to speak of (Schütz, 1989; Henker *et al*., 2007, 2008). Not all strains, however, were affected, and the mechanism(s) of resistance remain unclear. Nevertheless, it is not surprising to find such differences, as coevolution of gut microbial communities and pathogens make the latter evolve mechanisms to overcome resistance of commensals to invasion into or destruction of their niches. The differences in susceptibility to microcins among different classes of diarrhoeagenic *E. coli* are quite surprising, as all typical EPEC strains tested during this study were susceptible while all EAEC strains were resistant. Among other groups, however, both resistant and susceptible strains were found. Although the number of strains tested in each group was not high enough for a general conclusion about microcin susceptibility of specific *E. coli* pathotypes group, it's giving us a hint for further research on mechanism of action of EcN microcins. From two lab strains tested one was susceptible and the other one resistant. The susceptible strain was a derivative of *E. coli* B and the resistant one an *E. coli* K12 based strain. This can be a clue to possible mechanism(s) of microcins resistance as lab strains have known genetic background, and its analysis can help to reveal genes that might be involved. However, these analysis exceeded the scope and the time frame of this study and have to be performed in future investigations.

4.2. EPEC are able to infect human DC and monocytes and induce the generation of pedestals

This study also demonstrated that EPEC is able to infect human immune cells, such as human DC (primary cells) and a human monocytic cell line (THP-1). This infection causes a histopathological picture that closely resembles the one observed in epithelial cells (forms pedestals under Tir insertion site as it is happening during epithelial cells infection). This result points to an uniformity of pathways that EPEC exploit to cause disease as they are apparently not specific for epithelial cells at least with respect to Tir translocation and pedestal formation. As there was no difference between DC and THP-1 cells in response to

EPEC infection, for further experiments mostly THP-1 cells were used as primary human DC cells can be rather variable in their responses and, in addition, are more difficult to maintain.

4.3. EPEC affects the viability of human monocytes

In epithelial infections EPEC has been reported to induce cell death studied in various different epithelial cell lines. Therefore, we were interested to see whether this could also be observed for immune cells associated with defense mechanisms in the gastrointestinal tract. We observed that EPEC decrease the cell viability of THP-1 cells after 4 hours of infection. Cell death due to EPEC infection had been reported previously for the epitheloid HeLa cells and had been described to involve mainly apoptotic mechanisms (Abul-Milh *et al*., 2001). However, under the conditions used in this study EPEC-induced cell death of the monocytic THP-1 cells did not involve apoptotic cell death as cells were PI-positive but annexin V-staining negative. Acquired data indicate that pathways used by EPEC to cause cell death are apparently different between myeloid cell lineages and epithelial cells. To identify putative factors that might be responsible for cell death we screened selected EPEC mutants. From eight tested mutants (Δ*escN*, Δ*tir*, Δ*eaeA*, Δ*sepZ*, Δ*espD*, Δ*espB*, Δ*espF*, Δ*map*), only Δ*espD* and Δ*escN* were unable to cause cell death, which indicates that one of the factors that appears to be essential to induce cell death in THP-1 and/or DC is EspD. However, thus far it remains unclear, whether this effect is directly or indirectly induced by EspD. EspD has pore-forming abilities and was shown to be responsible for erythrocyte hemolysis caused by EPEC (Warawa *et al*., 1999; Ide *et al*., 2001). However, proving EspD's direct role might be difficult as it is clearly needed for the translocation of EPEC T3SS-dependent effectors. In the study of Abul-Milh *et al*. factors responsible for cell death were found to be BFP and intimin (Abul-Milh *et al*., 2001). Thus far, in this study we have not tested BFP mutants as we focused mainly on LEE encoded effectors that are actually injected into the target cells. However, we also included an intimin mutant as intimin is directly involved in the interaction of Tir with bacterial cells serving as its ligand. We found the *eaeA* mutant (Δ*intimin*) to be able to cause

cell death to the same extent as the wild type strain (Fig. 14), although in comparison to the effect of the wild-type strain cell death was delayed. This delay was observed also with all other mutants investigated. Interestingly, specifically the Tir mutant of EPEC strain E2348/69 was found to cause more cell death than the wild type strain after 5 hours accompanied by enhanced LDH release from the infected cells than found with the wild type strain after 7 hours. It had been previously observed that under low Ca^{2+} conditions Tir expression is induced and EspB and EspD are secreted in lower amounts (Ide *et al*., 2003). If the medium is supplemented with Ca^{2+}, the Tir secretion is reduced and more EspB and EspD is secreted (Ide *et al.*, 2003). It can be speculated that there is a direct correlation between amount of Tir, from one side, and EspD and EspB, from the other. If this hypothesis is true, in the Tir mutant there should be more EspB and EspD protein secreted, which is further enhancing EPEC-mediated cell death. Interestingly, several mutants (Δ*sepZ*, Δ*espB*, Δ*espF*, Δ*map*), although they were not able to kill as many cells as the wild type strain, induced cell death to a significantly higher extent than Δ*espD* and Δ*escN*. For *sepZ* mutants, a number of effects normally caused by EPEC were shown to be delayed. The fact that *espF* and *espB* mutants are unable to kill the cells as efficiently as the wild type strain is probably connected to the inability of these mutants to block phagocytosis. As the MOI used was 20, phagocytosis - especially in the initial stages - can significantly reduce the number of bacteria. The inability of the *map* mutant to cause the same viability decrease as the wild type strain is puzzling and might be connected with an activity of this particular effector specific for phagocytes as all previously reported effects appear to be either specific for epithelial cells (microvilli and tight junctions disruption) or irrelevant to necrotic cell death. The observation of cells infected with different EPEC mutants revealed some unexpected differences between the mutants. According to current opinion *espD* and *espB* mutants are deficient in secretion and therefore were expected to behave similarly as the *escN* mutants or any other secretion deficient mutants. However, all three mutants induced different phenotypes while interacting with THP-1 cells. The *escN* mutant was not able to kill cells and did not induce substantial cellular aggregates. The *espD* mutant was not able to kill the cells either, but almost all eukaryotic and bacterial cells were found in large aggregates. The ability of the *espB* mutant to cause

cell death was intermediate between uninfected cells used as controls and the *escN* and *espD* mutants. The EspB mutant also caused bacterial and eukaryotic cell aggregation in the same way as the *espD* mutant. However, the underlying mechanisms are thus far unknown and have to be addressed in future studies.

4.4. EcN is able to prevent cell death in co-infections with EPEC

The addition of EcN to the THP-1 cells together with EPEC E2348/69 completely abolished the detrimental effects of EPEC on cell viability. As expected from our preceding experiments regarding the sensitivity of EPEC towards microcins we could clearly show that under co-infection conditions the protective effect of EcN was associated with the secretion of microcins, as neither SK22D (microcins-negative EcN mutant) nor C600 were able to prevent the observed decrease in viability.

4.5. EPEC inhibits its own phagocytosis by human DC

For being successful pathogens intestinal bacteria should have evolved strategies to overcome host defense systems. First of all they should be able to attach to the surface of epithelial cells to prevent removal by peristalsis. Pathogens should also protect themselves against defensins and S-IgA. In their defense against immune cells many pathogens are actually able to either block phagocytosis or to survive inside the phagocytes (Buchmeier and Heffron, 1991; Rosqvist *et al*., 1988; Tilney *et al*., 2001). EPEC was shown to block its own phagocytosis and also opsonophagocytosis of red blood cells by J774 cells. As J774 cells are of murine origin we investigated this effect in a more relevant model with human immune cells. Furthermore, we were interested to investigate also a probiotic strain for putative inhibition of phagocytosis, as it would certainly also be beneficial for commensals to escape

phagocytosis. We could show in this study that EPEC prevented phagocytosis by DC. Almost no GFP-positive cells were found in infections with EPEC expressing GFP, whereas GFP-labeled EcN were phagocytosed at a similarly high rate as C600. Differences between control cells and EPEC infected cells were never more than 5% whereas difference between EPEC and C600 (or EcN) infected cells could be as much as 100% and even more. Blocking its phagocytosis by DC could potentially be important for EPEC because DC are usually the first phagocytes to take hold of the bacteria at the intestinal barrier. Since EcN uptake-indices were only a little bit lower than those for C600 it can be concluded that EcN does not affect phagocytosis by DC.

4.6. Infection of human DC and human monocytes by EPEC or EcN results in different gene expression profiles

4.6.1. Changes in gene regulation by EPEC and EcN infected cells

Numerous studies addressed the immunomodulation by EPEC in epithelial cells, however, data addressing putative immunomodulatory effects of EPEC on human immune cells are scarce. Therefore, to study global changes in gene expression of immune cells during infection microarray analysis were performed. For the DNA-microarray analysis DC or THP-1 cells were infected with either EPEC E2348/69 or EcN. These experiments should give a first indication on the ability of antigen-presenting cells to fine-tune their response depending on the 'danger' of bacteria while there are no other cell types available to 'instruct' them. Moreover, it should also be possible to obtain preliminary evidence on putative immunomodulatory capabilities of exemplary commensal and pathogenic bacteria. Since in our experiments we employed *E. coli* most of the genes were likely to be regulated in the same manner (up or down) as a common response to *E. coli* infection. Indeed, for THP-1 cells out of 1053 genes that passed validation, 944 were regulated in the same direction as a

response to EcN or E2348/69 infection. For DC only 48 differently regulated genes were found out of total amount of 972. Interestingly, for both cell types most of the differentially regulated genes were upregulated in EcN and downregulated in EPEC.

In DC only seven genes were down-regulated in EcN but upregulated in EPEC, and only 1 is coding known protein – adaptor protein of protein kinase A (AKAP10). Our interest was directed towards genes that were regulated differently, including genes that were regulated in the same manner, however, to a different extent. These genes were selected and divided into several artificial groups depending on their involvement in cellular processes (sometimes when gene is encoding a protein involved in several processes it was included in one group depending on which function was more interesting for this study).

4.6.2. Global transcriptome analysis in DC infected with EcN or EPEC E2348/69

Many interleukins genes were found to be regulated differentially. Many pathogens are known to modulate immune system functions on the level of cell-to-cell communication by cytokines. Interference with intercellular communication in the hosts' immune responses opens nearly endless possibilities for the pathogen to modulate immune responses for its benefit. Interestingly, all of the chemoattractant ligands/receptors studied except XCL1/XCL2 and CXCL11 (out of 10 found) were upregulated in EcN and downregulated or remained unaffected in EPEC, or were upregulated higher in EcN in comparison to cells infected with EPEC. The overall results obtained from transcriptome analysis experiments indicate that EPEC exert immunomodulatory activities which is in good agreement with the observation that EPEC-infected people do not develop severe intestinal inflammation (Ashkenazi *et al*., 1983). At the same time the upregulation of pro-inflammatory factors in EcN-infected cells is reasonable as any bacterium that did pass the epithelial layer should be recognized as potentially harmful. Therefore, in conclusion the results for EcN appear to represent the normal reaction of the immune cell whereas the results obtained with EPEC infected DC indicate a subversion of normal immune signaling by thus far unknown means.

In the following, some of the results obtained in the microarray experiments will be discussed in further detail. However, one should keep in mind that microarray analysis usually just reflects a momentary global profile of alterations in transcription (expression) which nevertheless might suggest important leads for future investigations. In addition, at present not all alterations observed in microarray analysis could be confirmed by independent methods due to lack of time.

Similar results were obtained for genes encoding proinflammatory cytokines, such as *IL-1A*, *IL-1B*, *IL-6*, *IL-23A,* and members of TNF superfamily except *TNFSF8* and *TNFSF14*. This can be also viewed in the context of inhibition of inflammation. *IL-7* was also detected in the array and was unaffected for EPEC infected cells but upregulated 2-fold following EcN infections. Also *IL-12* p40 was upregulated, but as this subunit can form homodimers that block signaling it is impossible to draw conclusions on putative consequences of such an upregulation without looking at *IL-12* p35 subunit expression - this, however, did not pass the validation of the array results and has to be further investigated in future studies. *IL-10* showed an overall predictable expression pattern as it was upregulated in EcN and not affected in EPEC. At the same time, suppressors of cytokine signaling genes (*SOCS3* and *SOCS4*) were expressed either similarly (*SOCS4*) or upregulated to a much higher extent in EcN infected cells (6.7 against 27.3 for *SOCS3*).

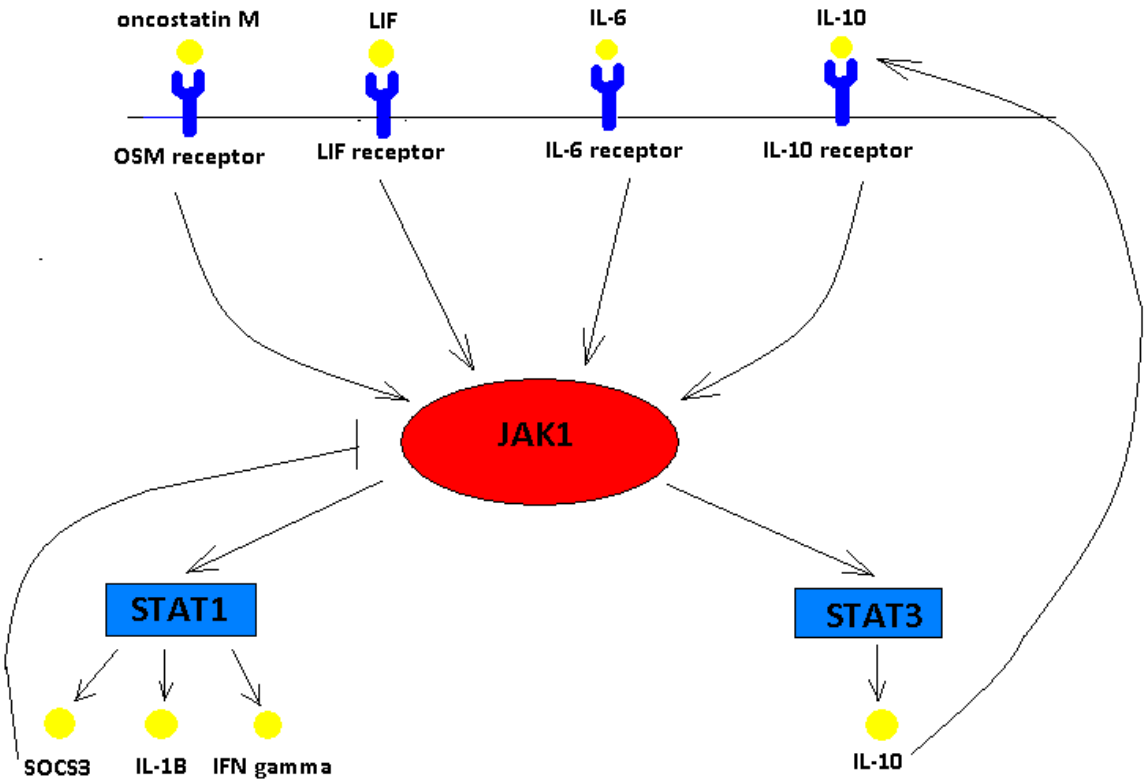


Fig.30. JAK/STAT pathway.

This result pointed to a putative involvement of signaling events via the JAK/STAT pathway in EcN infections (Fig. 30). All factors depicted in this scheme were found to be upregulated to a higher extent in EcN infected cells except IFNγ. Among the STAT factors, STAT5B passed validation in the array analysis and was upregulated to the same extent in EcN or E2348/69 infected cells. However, as the JAK/STAT pathway regulates many cellular processes the physiological relevance of these observations remains speculative.

4.6.3. DC – cytoskeleton/adhesion/migration

In this functional context genes of two proteins were found to be downregulated by EPEC and upregulated by EcN, namely collagen VIII α2 and the microtubule-associated protein 7 (MAP7). IQGAP2 and IQCG proteins were upregulated in EPEC infected cells stronger than in

EcN infected cells. MAP7, IQGAP2, and IQCG are probably involved in cytoskeleton rearrangements caused by EPEC.

4.6.4. DC – endocytosis/vesicular trafficking/phagocytosis

Interesting results were obtained for stonin 2 and septin 9 (SEP9). The former was upregulated in EPEC 2.8-fold in comparison to EcN 11.7-fold, the latter showed 13.1-fold downregulation for EPEC and 3.4-fold upregulation for EcN infected cells. The endocytic protein stonin 2 harbors multiple AP-2 binding sites and is involved in clathrin/AP-2 mediated endocytosis (Walther *et al*., 2003). However, it's unclear whether this process might play any role in the interactions of DC with EPEC and EcN.

Some speculations can be made on the role of SEP9. Septins are GTPases involved in a number of processes such as cytokinesis and secretion (Trimble *et al*., 1999; Kartmann and Rodd, 2001). SEP9 was also reported to be involved in bacterial entry into the host cell. Latex beads coated with *Listeria* surface protein InlB were localized in phagosomes with SEP9 (Mostowy *et al*., 2009). Such a pronounced downregulation in EPEC infection might be correlated to the ability of EPEC to block phagocytosis, while in EcN infected cells, where phagocytosis can occur, *SEP9* is upregulated.

4.6.5. DC – receptors/surface antigens

BAMBI showed a 13.8-fold upregulation for EPEC and 22.3-fold for EcN infected cells. BAMBI is a factor suppressing TGFβ signaling, as it is a receptor that is able to bind TGFβ but is unable to mediate downstream signaling (Sekiya *et al*., 2003). Therefore, it can be speculated that upon contact with bacteria DC are prone to become less responsive to TGFβ signaling. This might be important in the gut as this cytokine is involved in tolerance induction.

TLR1 was downregulated in both EPEC (10.8-fold) and EcN (2.6-fold) infected cells. This receptor is recognizing bacterial peptidoglycan and lipopeptides. Downregulation might support a reduction in the activation of inflammatory pathways.

4.6.6. DC – kinases/phosphatases/gene expression regulators

Genes associated with this functions were used to get an insight into the pathways that might be involved in response to infection with both strains. However, while studying cellular signaling cascades it is very helpful to look not only on transcription but also on post-translational activation events such as phosphorylation. So data from microarray analysis for these proteins should be taken only as a hint for further research. Most of the genes in this group showed comparable regulation upon EPEC and EcN infections. Early growth response 1 (*EGR1*) was upregulated in EcN infected cells twice as much as in EPEC infected (10.9 against 4.9-fold). This factor is activated by phosphorylation through ERK1/2 activated in a signaling cascade that also involves SYK kinase. It is also phosphorylated by MAPK14. This protein is a zinc-finger motif-containing transcription factor, so after activation it is targeted to the nucleus. EGR1 is involved in an early response to growth factors, and participates in proliferation and differentiation of cells from the myeloid lineage. Two ways of EGR1 activation are depicted in Fig. 31.

IL-1 receptor associated kinase (*IRAK2*), also showed higher upregulation in EcN infected cells (10.3 against 3.8-fold). This kinase is involved in several cellular processes including inflammation via the activation of NF-κB. It is activated through IL-1 receptor. On Fig. 32 part of the NF-κB pathway is shown which includes IRAK.

TNF receptor associated factor 1 (TRAF1) is activated through RIPK2 that can be recruited for example by CARD domain-containing proteins or through FADD containing proteins. In the mouse model there is no apparent phenotype of $TRAF1^{-/-}$ mice, but some abnormalities were observed in T cells, which were hyperproliferative upon TCR stimulation with anti-CD3 antibodies (Tsitsikov *et al*., 2003).

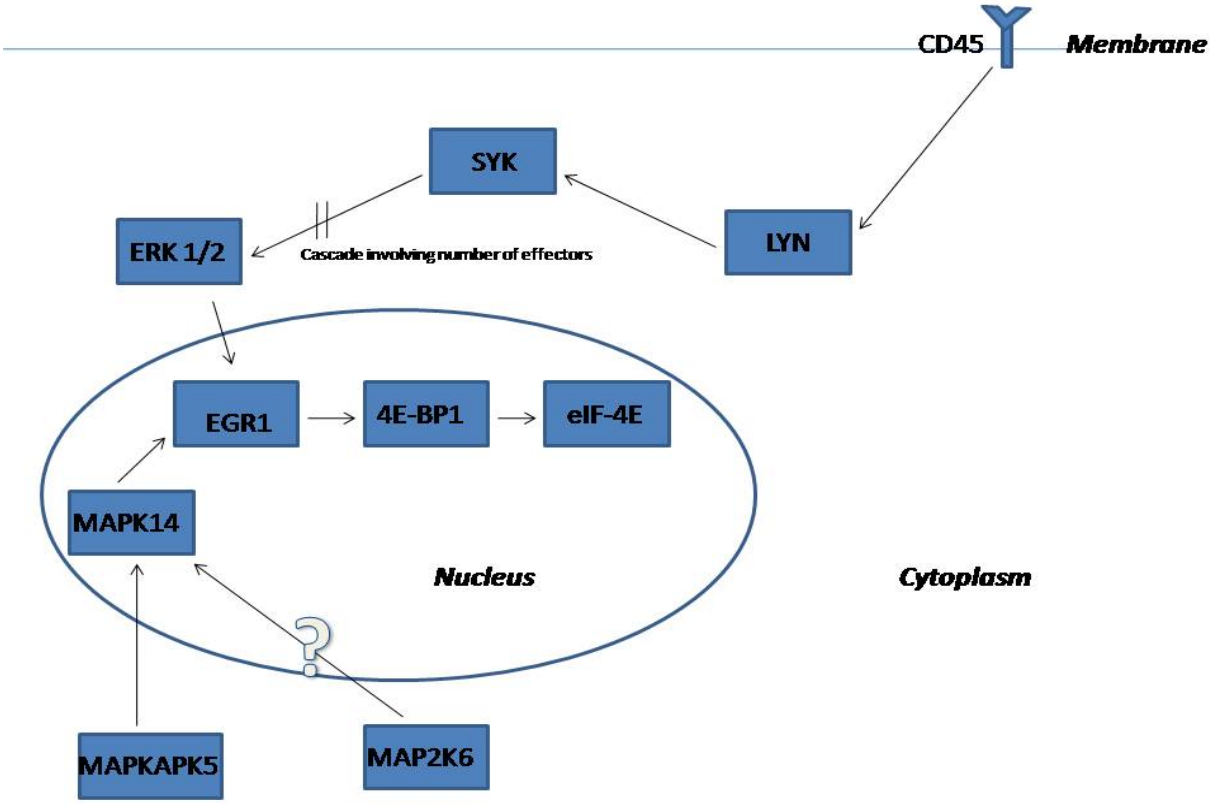


Fig. 31. Scheme of EGR1 activation. Obtained using Linnea™ Pathways (Invitrogen).

In DC the balance between the activation of caspases and pro-survival stimuli is regulated to a certain extent by TRAF1. The physiological role of TRAF1 is at least partially mediated via its ability to regulate TRAF2, as in TRAF1$^{-/-}$ DC TRAF2 is depleted and balance tilts from survival to apoptosis (Aaron *et al*., 2002). According to Oyoshi *et al.* the role of TRAF1 was also shown for the recruitment of lymphocytes, DC, and monocytes to the lung airways upon treatment with LPS (Oyoshi *et al*., 2006). The reported roles of TRAF proteins in survival (through NF-κB activation) are summarized in Fig. 32 and Fig. 33.

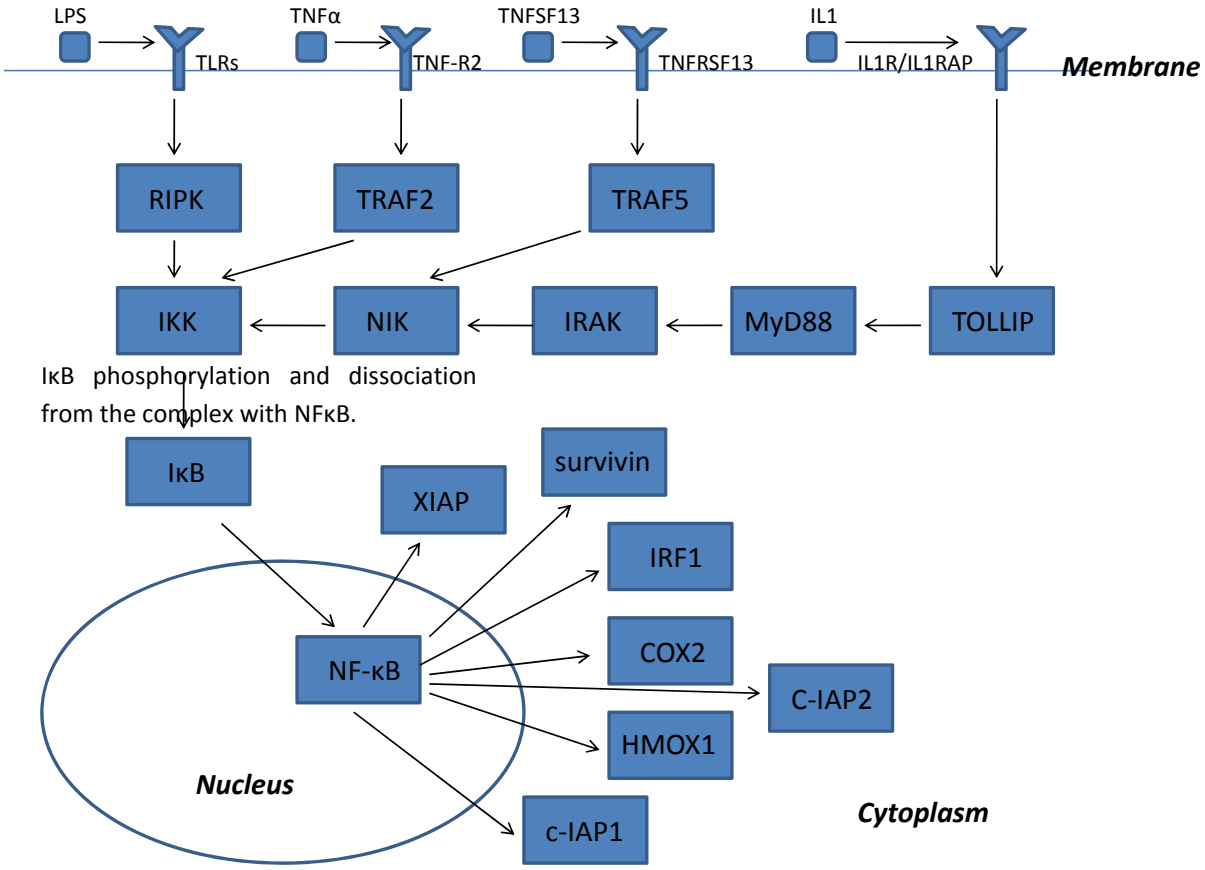


Fig. 32. Scheme of NFκB pathway (incomplete). Obtained using Linnea™ Pathways (Invitrogen).

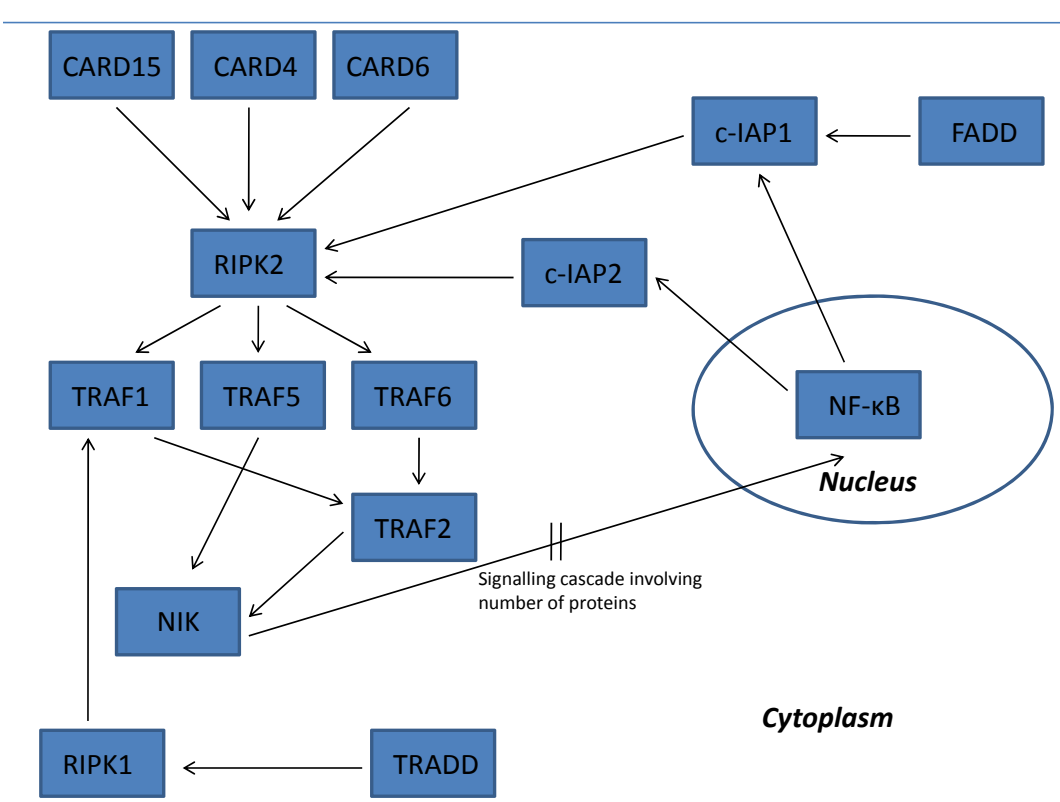


Fig. 33. Scheme of the involvement of TRAF proteins in promoting survival. Obtained using Linnea™ Pathways (Invitrogen).

4.6.7. Alterations in the expression of immune response genes in THP-1 cells

Just like in DC also in THP-1 cells the expression of almost all pro-inflammatory cytokines was enhanced in EcN infected cells compared to EPEC infected cells. Exceptions were genes for interferons α7 and α10 (fold of regulation respectively for EcN and EPEC: NA – 2.2 and -4.5 – 3.6), CCR1 (fold of regulation respectively for EcN and EPEC: -2.2 – NA), CXCR7 (fold of regulation respectively for EcN and EPEC: NA – 2.8), CCL14/CCL15 (fold of regulation respectively for EcN and EPEC: -3.2 – 3.2), CCR9 (fold of regulation respectively for EcN and EPEC: 2.3 – 4.5), TRAF3 (fold of regulation respectively for EcN and EPEC: NA – 2.3), TNFSF12

(fold of regulation respectively for EcN and EPEC: -2.2 – NA), TNFSF14 (fold of regulation respectively for EcN and EPEC: -5.2 - -2.2).

Interestingly, the *IL-17C* cytokine gene was also found to be upregulated in EPEC infected cells. IL-17 is now recognized as one of the most important players in inflammatory responses, as it is secreted by activated Th17 cells and as this cytokine with other cytokines produced by Th17 cells causes strong inflammatory responses its production is regulated tightly (Conti et al., 2009; Korn *et al*., 2009). However, as upregulation was only 2.3-fold compared to the control further investigations are needed to elucidate whether this cytokine is actually produced also by monocytes and not only by activated T cells.

IL-8 was upregulated in cells infected with either of the two *E. coli* strains. Expression was enhanced more in EcN infected cells than in EPEC infected (325-fold against 163-fold respectively). However, with IECs it was shown that EPEC actually suppress IL-8 secretion. So the acquired data suggests that a similar regulation might take place also in the myeloid cell lineage.

4.6.8. Changes in the expression of cytoskeleton/adhesion/migration associated genes in THP-1 cells

One of the interesting genes in this group of cytoskeleton/adhesion/migration related genes in THP-1 cells showing a different expression level related to the infecting strain is *ITGA9* (integrin alpha-9/beta-1). ITGA9 is involved in cell-cell and cell-matrix adhesion. Expression of *ITGA9* was not affected in EcN infected cells but 6-fold downregulated in EPEC infected THP-1 cells. This result can give a further clue why EPEC infected cells cannot adhere well to tissue culture surfaces. ITGA9 also functions as a receptor for VCAM1, cytotactin, and osteopontin.

Contactins 1 and 3 (CNTN1 and CNTN3), adhesion molecules of the immunoglobulin family, that were first described in neurons, were not affected in EPEC infected cells and upregulated in EcN infected (13-fold for *CNTN1* and 7-fold for *CNTN3*). It should be noted that almost all

adhesion molecules studied showed upregulation for EcN while their expression in EPEC infected cells was not changed or even downregulated.

4.6.9. Alterations of endocytosis/vesicular trafficking/phagocytosis associated genes in infected THP-1 cells

Matrix metalloproteinase 12 (*MMP12*) or macrophage elastase is a secreted proteinase involved in extracellular matrix degradation. However, recently it was also shown to kill bacteria in murine macrophages after its recruitment into phagolysosome. The effect was proven to be direct, as the recombinant protein also had a bactericidal effect (Houghton *et al*., 2009). The expression of elastase is not changed in EcN infected cells, however, was found to be strongly upregulated in EPEC infected THP-1 cells (8-fold). This might be interpreted that only pathogenic bacteria enhance the expression of *MMP12*. It could also be speculated that the overexpression would correspond to MMP12 secretion and not to its recruitment into the phagosome as there not much phagosomes formed in EPEC infected cells because the bacteria are inhibiting phagocytosis.

4.6.10. THP-1 - kinases/phosphatases/gene expression regulators

For THP-1 cells as well as for DC most of the genes associated with these functions were regulated similarly in EPEC and EcN infected cells. Among the relatively few exceptions were *EGR1* (17.8-fold in EcN infected cells against 8.6-fold in EPEC infected), *MAP2K6* (9.9 against 3.1-fold), *NFKBIB* (16.5 against 4-fold), *SMAD2* (8.1 against -2.3-fold) and *TRAF5* (-2.1 against -4.4-fold).

MAP2K6 (MKK6) phosphorylates MAP kinases, which are involved in many cellular functions. Involvement of MAP2K6 in specific processes in monocytes was not reported so its function in response to infection remains elusive.

NF-κBIB comprises IκB protein that inhibits NF-κB by direct binding in the cytoplasm. A schematic overview of NF-κB signaling is presented in Fig. 32. This result suggests a remarkable reduction in NF-κB activation by EcN.

SMAD2 is activated by TGFβ receptor kinase in response to TGFβ receptor ligation. After activation it is targeted to the nucleus and participates in transcription activation. In mouse model it was shown that SMAD2$^{-/-}$ mice show spontaneous dedifferentiation of hepatocytes in the liver (Ju *et al*., 2006).

TRAF5 is involved in numerous cellular pathways including apoptosis suppression in a cascade resulting in activation of RelA. TRAF5 can be activated for example through TNFRSF8 and TNFRSF13. TRAF5 can also be activated by RIPK1. Involvement of TRAF5 in cellular pathways can be understood from Fig. 32 and Fig. 33.

4.6.11. THP-1 cells - apoptosis related genes

Just as had been found for the analysis of the DC transcriptome by microarray in the analysis of THP-1 cells no genes commonly associated with apoptosis were found to be upregulated. To the contrary, these genes were either not affected or downregulated. *BOK* (BCL2-related ovarian killer), which codes for a pro-apoptotic protein was downregulated 4-fold in EcN and not affected in EPEC infection; *BAG4* (Bcl-2-associated athanogene 4), which codes for an anti-apoptotic product was not affected in EcN infected cells and downregulated 3.4-fold in EPEC infected. BAG4 was shown to prevent constitutive TNFRSF1A signaling. *BAD* (BCL2-antagonist of cell death protein) is a pro-apoptotic protein. Its activity is regulated through the AKT and MAP kinases, as well as through the protein phosphatase calcineurin. The gene encoding the pro-apoptotic protein THAP3 (THAP domain containing, apoptosis associated protein 3) was not affected in EPEC but downregulated 5-fold in EcN infections.

In summary all genes investigated here encoding pro-apoptotic proteins were downregulated in EcN whereas they were not affected in EPEC infected cells. The BAG4 gene encoding an anti-apoptotic protein showed a reverse regulation. Possible consequences from

such a regulation could be an apoptosis inhibition by EcN and apoptosis induction by EPEC, however, the monitoring of possible apoptotic processes by staining with anti-annexinV antibodies actually did not provide evidence for apoptosis. This could mean that either other pathways intervene with induction/inhibition of apoptosis through the above mentioned factors or that in control cells they are expressed in such a low level that downregulation of them cannot influence any signaling pathways in the cell.

4.6.12. Comparison between DC and THP-1 expression profiles

It was interesting to compare the expression of proteins involved in inflammatory responses in THP-1 cells and DC. In general, with a few exceptions, the changes in gene expression appeared to be more profound in THP-1 cells than in DC.

4.6.12.1. Immune response

It can be speculated that as DC are well suited for fine-tuning of immune responses mainly by antigen presentation they might be a able to produce less chemoattractants involved in strong inflammation. However, at the same time DC are upregulating strongly the expression of the CXCL11 chemokine which attracts T cells and of CCL20, a chemoattractant for lymphocytes. A further exception is the regulation of *IL-6*, which was strongly upregulated in EcN in comparison to EPEC infected cells. IL-6 was shown to have specific functions in DC, in blocking NF-κB signaling and the CCR7 production. For THP-1 cells upregulation wasn't so profound, 3.6-fold for EPEC infected cells and 4.5-fold for EcN infected, and there were only slight differences between the two strains.

IL-1A and *IL-1B* were found in the array analysis for both DC and THP-1 cells, and for both cell types they were upregulated to a higher extent for EcN infected cells than for EPEC infected cells. For THP-1 cells the factors of upregulation were 21.6 vs 7.3 for *IL-1A* for EcN and EPEC respectively and 217.2 vs 61.1 for *IL-1B*, for DC – 13.2 vs 1.99 (which was considered as unaffected) and 6.1 vs 3.8.

TNF-α is one of the key proinflammatory cytokines exerting a plethora of effects during inflammatory and pathologic processes. For instance, TNF-α is cytolytic or cytostatic for many tumor cells, induces the terminal differentiation and the synthesis of G-CSF in monocytes, and acts as a mitogen for B-lymphocytes. In neutrophils TNF-α induces the production of reactive oxygen species, serves as a chemoattractant for these cells, increases phagocytosis and adhesion to the endothelium. In THP-1 cells it was upregulated after infection with either strain (8.7-fold in EPEC infected vs 18.1-fold in EcN infected), and in DC it was downregulated in EPEC infected and upregulated in EcN infected cells (-2.2 vs 5.4-fold).

TNFSF14 is a member of the TNF superfamily, which acts as a costimulatory factor for lymphocyte activation, stimulates the proliferation of T-cells and triggers apoptosis in various tumor cells. It shows a drastic difference in expression between THP-1 and DC, as in DC it was upregulated 129.2-fold for EPEC infected, 116.5-fold for EcN infected cells. However, in THP-1 cells this gene was found to be downregulated 2.3-fold in EPEC and 5.1-fold in EcN.

4.6.12.2. Receptors/surface antigens

Among other notable genes is the gene coding for the Toll-like receptor 1 (TLR1), which recognizes peptidoglycan and bacterial lipopeptides. It was downregulated strongly in infection with EPEC and was found to be either not affected (THP-1) or slightly downregulated (DC) in infections with EcN. A physiological relevance of such a downregulation might be to inhibit the activation of inflammatory pathways in order to prevent excessive tissue damage.

4.6.12.3. Endocytosis/vesicular trafficking/phagocytosis

SEP9 was found to show a quite different regulation in THP-1 cells and DC. This gene was downregulated in THP-1 cells infected with EPEC (2.8-fold) but upregulated in DC infected with EPEC (3.4-fold). In EcN infected THP-1 cells *SEP9* was not affected, however, in EcN

infected DC this gene was strongly downregulated (13.1-fold). Possible reasons for such a different regulation are not quite clear.

4.6.12.4. Kinases/phosphatases/gene expression regulators

Analysis of cellular pathways with Pathway Architect software from Gene Spring revealed only one pathway significantly affected in both THP-1 and DC. A schematic overview the pathway is presented on Fig. 34.

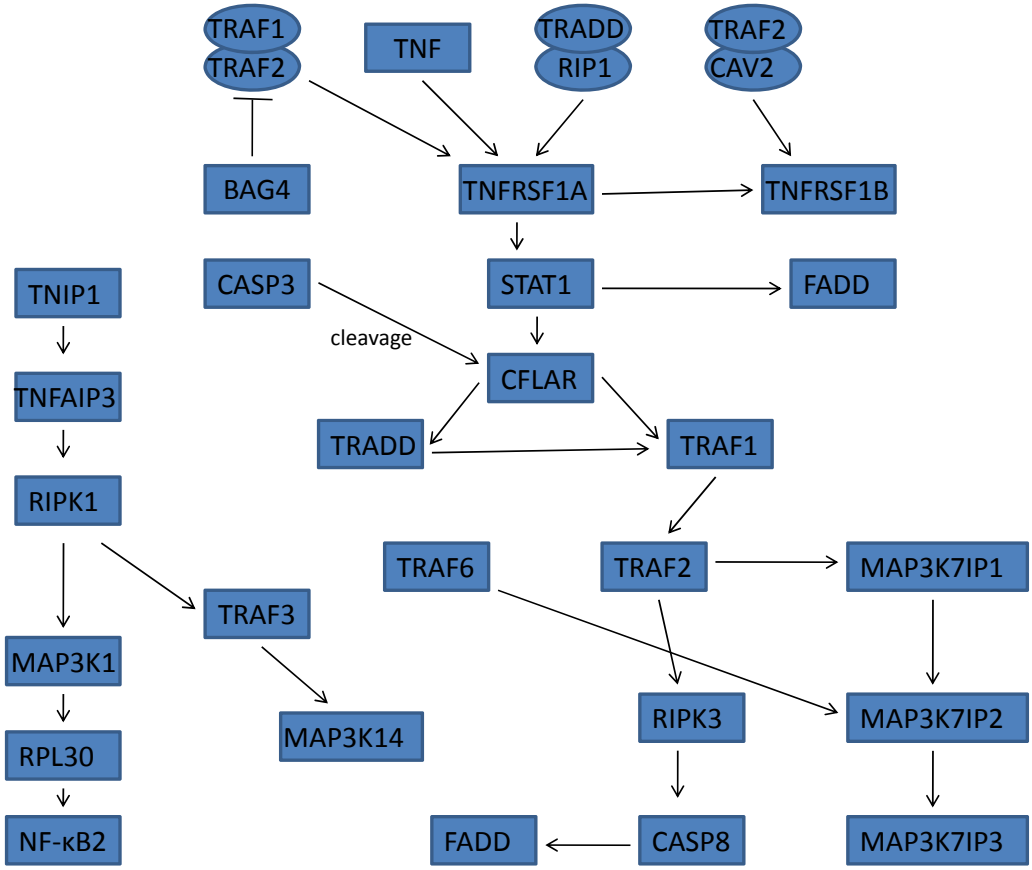


Fig. 34. TNF-α pathway scheme. Obtained using Pathway Architect software (Gene Spring 10.0).

Table 13 represents the regulation of various different genes (fold changes) represented in the scheme found in the gene expression arrays for THP-1 cells and DC.

THP-1	*EPEC*	*EcN*	*DC*	*EPEC*	*EcN*
TNF	8.7	18.1	TNF	-2.2	5.4
MAP3K7I	4.3	8.4	MAP3K7	2.5	3.4
TRAF1	6.1	4.1	TRAF1	6.3	13.9
TRAF3	2.3	2	TRAF3	-2.8	-4.4
BAG4	-3.4	NA	BAG4	-2.2	-2
CFLAR	2.5	4	CFLAR	2.3	2
MAP3K7I	NA	2.2	CASP8	-3.1	-3
CASP3	NA	2.1	TRADD	-2.2	-2

Table 13. Expression of genes involved in TNF pathway as was assessed by gene expression microarray.

These results might suggest that the branch of the pathway which starts from TNF-α binding to TNFRSF1A is upregulated in THP-1 cells infected with either of the two *E. coli* strains and also in EcN infected DC. In EPEC infected DC, however, TNF-α showed a slight downregulation. For THP-1 cells also the gene for the downstream protein CFLAR showed a similar upregulation (higher upregulation in EcN infected cells than in EPEC infected cells). However, for DC *CFLAR* showed an upregulation in infections with either strain. TRADD that is downstream of CFLAR showed downregulation in infections with either strain. The branch of the pathway starting from BAG4 and leading to the inhibition of TNFRSF1A signaling seems to be either downregulated or not affected in both cell types.

Based on these comparisons it is obvious that THP-1 cells and DC have substantial differences in their responses to infections. However, it should be kept in mind that most of the proteins in signaling pathways are activated by phosphorylation or cleavage and transcriptional regulation study cannot provide a hint on their actual activity.

4.6.12.5. Apoptosis

BCL2L11 was found in both arrays (for THP-1 cells – 2.5-fold in EPEC infected but not affected in EcN infected cells, for DC – 2-fold downregulation for EPEC infected and 2.9-fold downregulation for EcN infected cells). The BCL2L11 protein belongs to the BCL-2 protein family. Members of this family form hetero- or homodimers and act as anti- or pro-apoptotic regulators that are involved in many cellular processes. BCL2L11 interacts with BCL2, BCL2L1/BCL-X(L), MCL1, and is a pro-apoptotic protein. Evidence exists that BCL2L11 plays a role in neuronal and lymphocyte apoptosis. Studies with transgenic mice suggested a function of this gene as an essential initiator of apoptosis in thymocyte negative selection (Bouillet *et al*., 2002).

The *BCL6* gene codes for a protein that acts as a sequence-specific repressor of transcription, and that has been shown to modulate the STAT-dependent interleukin 4 (IL-4) responses of B cells. This protein acts as an anti-apoptotic and prevents the increase in reactive oxygen species in lymphoma cells (Kurosu *et al*., 2003). In the array it was upregulated in THP-1 cells irrespective of the bacterial strain used for infection (16.4-fold and 23.8-fold for EPEC and EcN infected cells, respectively) and either not affected (for EPEC infected cells) or 2.4-fold upregulated (for EcN infected cells) in DC.

In conclusion, the results obtained with microarray gene profiling experiments indicate that antigen presenting cells are to some extent able to differentiate their responses, distinguishing between ‘useful’ bacteria that should not cause inflammatory responses and pathogens which should be eliminated. Furthermore, it should be kept in mind that responses of eukaryotic cells to pathogens are subject to constant co-evolution as hosts always intend to detect and eliminate pathogens as fast as possible and pathogens always need to develop new ways to undermine and subvert the hosts defense mechanisms. This dynamic balance is maintained by influencing different cellular processes by various means such as using secreted proteins or surface structures or by evolving mechanisms of resistance to host defense systems. An example for these strategies has been characterized in this study as our results indicate that EPEC intervene with some global cellular pathways resulting in the reduction in the expression of various genes.

4.7. The secretion of proinflammatory cytokines in EPEC infected cells is lower than in cells infected with EcN

To study changes in cytokine expression on the protein level the 'RayBio inflammatory array' was employed. Three groups of cytokines can be differentiated based on the different degree of upregulation.

The first group includes cytokines that were upregulated up to 10-fold. Among these cytokines IL-1A, IL-3, and IL-11 didn't change their expression (if the 2-fold lower limit for upregulation is selected as it was done for the microarray). The former two cytokines have pro-inflammatory activities while the latter is an anti-inflammatory interleukin.

G-CSF, which promotes survival, differentiation, and activity of neutrophils, showed slight upregulation only for EPEC infected cells. However, as upregulation was only 2,1-fold, while EcN showed 1.8-fold induction, this result can be taken as an indication of comparable regulation for both strains.

IL-8 was upregulated much stronger in C600 and EcN infected cells. This can be a further clue indicating immunosuppressive ability of EPEC.

The strongest upregulation in this group was detected for TIMP-2 (tissue inhibitor of metalloproteinase 2) cytokine, which was around 4-fold for EPEC and EcN, and around 6-fold for C600. This cytokine inhibits metalloproteinases (MMPs), which are enzymes involved in extracellular matrix degradation and remodeling. Their role was shown in development of such pathological conditions as rheumatoid arthritis and periodontal diseases (Birkedal-Hansen *et al*., 1993). So the upregulation of TIMP-2 can be directed to a decrease of inflammation decrease, which is usually associated with high expression of MMPs. EPEC was found by microarray analysis to cause a strong induction of MMP-12. Although it has not been elucidated whether TIMP-2 has an effect towards this specific metalloproteinase, the enhanced TIMP-2 expression by EPEC infected cells might possibly counterbalance this enzyme during infection.

In the second group (upregulation from 10 to 35-fold) for IL-12p40, IL-12p70, and I-309 EPEC infected cells showed no upregulation, while both C600 and EcN infections caused upregulation of these cytokines. The strongest upregulation of both whole IL-12 and IL-12 p40 were observed in C600 infected cells, I-309 was upregulated the most in EcN infected cells (5-fold against 15-fold). Levels of IL-12p40 were in agreement with RNA profiling, as for p40 there was strong upregulation for EcN and C600 and no change in the expression for EPEC. IL12p70 levels in supernatants again suggested immunosuppression by EPEC.

The I-309 cytokine is chemotactic for human monocytes and also leads to transient increase in cytoplasmic Ca^{2+} levels in those cells.

Eotaxin-2 was upregulated slightly (around 3-fold) for EPEC and for C600 and EcN upregulation was more profound (8 and 12-fold). Eotaxin-2 is a C-C motif cytokine (also known as CCL24) which induces chemotaxis of eosinophils and basophils.

For IL-10 results were generally in accordance with Microarray data and RT-PCR, as it was upregulated to a higher extent in EcN and C600 than in EPEC infected cells.

In third group 2 cytokines were found to be upregulated more than 100-fold, namely IL-1B and IL-6. IL-1B, a proinflammatory cytokine, was upregulated to the same extent regardless of the strain used for infection, but IL-6 showed differential regulation. This cytokine showed much stronger upregulation in C600 and EcN infected cells. Although this cytokine is mainly recognized as proinflammatory, it was also shown to block NF-κB activity in DC. So from this point of view it is understandable why this cytokine expressed higher in EcN and C600 infected cells than in EPEC infected.

4.8. Expression of IL-10 and IL-12 cytokines by qRT-PCR

The balance of IL-10/IL-12 in response to bacterial infections can hardly be overestimated with respect to driving responses either to tolerance or to inflammation. The former is recognized as an anti-inflammatory cytokine while the latter is a cytokine with pro-inflammatory properties. IL-10 is used by many bacterial and viral pathogens to avoid

active responses to their invasion. In healthy organisms the IL-10 response is generated towards microbiota and in inflammation IL-10 levels tend to decrease. IL-12, on the other hand, is upregulated during the course of infection. Phagocytes tend to respond with IL-12 secretion to contact with any bacteria. That means that both IL-12 and IL-10 are upregulated in response to commensal bacteria, though the former is induced to a smaller extent. Reaction to a pathogenic organisms only involves strong IL-12 upregulation. IL-10 is necessary for Treg cell activation. Expression level of both of these cytokines by DC were found to be altered in gene expression array analysis. As expected, IL-10 was not affected by EPEC infection, but was found to be upregulated 10-fold by EcN. In the array only IL-12p40 was found, which was not affected by EPEC but upregulated 2.7-fold by EcN. As p40 subunits can form homodimers that bind to receptor but do not generate downstream signaling, it is feasible that overexpression of the p40 subunit over p35 can actually reduce inflammatory responses by blocking part of the IL-12 receptors. To investigate further IL-10 and IL-12 expression in DC infected with EPEC and EcN qRT-PCRs were performed. For IL-10 the qRT-PCR data were in agreement with the microarray results as it was upregulated in EcN 20-fold and downregulated in EPEC 5-fold. For IL-12 the p35 subunit was used, in contrast to the array where only the p40 subunit passed the validation. In contrast to the p40 subunit which was upregulated by EcN 2.7-fold and not affected in EPEC, the p35 subunit by qRT-PCR was upregulated for EPEC 4-fold and for EcN 2-fold. This might have been predicted knowing that EPEC is a pathogen supposed to turn on the inflammatory response and EcN is a probiotic bacteria that should not cause too much of inflammation.

4.9. Expression of micro-RNAs

As there is a growing evidence that miRNAs are involved in immune reactions regulation (Lindsay et al., 2008) we checked if miRNAs are differentially expressed in human monocytes infected with *E. coli* strains. For that THP-1 monocytic cell line was infected with *E. coli* Nissle 1917, *E. coli* C600, or enteropathogenic *E. coli* E2348/69. For all miRNAs found affected there

was strong upregulation in cells infected with C600. Reasons for such an upregulation are unclear, but it can be suggested that as C600 has no abilities for immunomodulation it non-specifically induced certain pathways involved in antibacterial response which resulted in expression of many genetic loci including the ones coding miRNA and anti-inflammatory proteins to block undesired inflammation that can result in cell and tissue damage. Among miRNAs differently regulated in EPEC and EcN infected cells were MIR-137 (NA – 3-fold respectively), MIR-98 (2.2 and 4-fold), MIR-548A-3P (NA – 3.1-fold), MIR-633 (2.2 – 4.9-fold), MIR-9 (NA – 3.6-fold), MIR-32 (NA – 3.7-fold), MIR-513B (NA – 3.4-fold), MIR-542-3P (2 and 4.8-fold), MIR513A-3P (4 and 2.8-fold), MIR-937 (4.4 and 3.2-fold). Most of the miRNAs here were not affected or slightly upregulated in EPEC infected cells and slightly upregulated (but higher than in EPEC infected cells) during EcN infection. Such results support a hypothesis that the EPEC immunosuppressive effect is mediated through a global pathway affecting among other genes also miRNA coding loci. Mainly attention was paid to the targets that were found in gene expression array to compare transcription profiles. Among MIR-137 targets there were found PALM2-AKAP2 (-2-fold for EPEC infected cells, not affected in EcN infected in gene expression array), CD83 (17.6 against 24.4-fold upregulation), DUSP4 (9.9 against 4.2-fold upregulation). For MIR-98 among targets were such genes as CNTN3 (NA – 6.9-fold), MAP4K4 (2.4-fold – NA). MIR-548A-3P affected such genes as SMAD5 (-2-fold – NA), SOCS4 (NA – 2-fold), CXCR7 (2.8-fold – NA). MIR-633 has such target genes as CPEB2 (4.3 against 2.2-fold), IL1RAP (4.2 against 5.2-fold). For MIR-9 there is only one target genes which was present on the gene expression array, namely CLDN18 (2.4-fold – NA). MIR-32 binds such genes as CPEB2, CD69 (4.6 – 11.1-fold), BCL2L11 (2.7 – 4.2-fold). Among MIR-513B targets only SMAD2 (-2.3 – 8.1-fold) was also present on gene expression array. MIR-542-3P is targeting PALM2-AKAP2, IL1RAP. Among MIR513A-3P targets are SMAD2, SMAD5 (-2 – NA), CD44 (7.4 – 6.6). For MIR-937 there is no targets found which would also be present in gene expression array. Though it's impossible to make a conclusion about protein expression from these RNA-based arrays (miRNA and gene expression array), it can be speculated that for many genes which are upregulated higher in EcN infected cells than in EPEC infected that difference will be partly eliminated on translational level as miRNAs will also be higher

upregulated. And as most of the genes in the gene expression array were upregulated higher in EcN infected cells, the picture of gene expression on protein level can change drastically due to appearance of a new player – miRNA.

4.10. Responses of intestinal barrier model to *E. coli* infection

We observed that in EcN infected cells numerous protrusions generated by DC were found to find their way through the filter and the Caco-2 epithelial layer. Next we checked whether EcN supernatant would have the same effect as live EcN. We were also able to show by scanning electron microscopy that the supernatant of the probiotic strain activated DC to form longer protrusions. This might facilitate an efficient passage of these protrusions to the apical side. Occasionally we could observe bacteria in close contact with DC on apical side (Fig. 28B) (presumably that picture is showing first stage of uptake of EcN bacteria by DC). However, on SEM images we were not able to detect as many protrusions on the apical side of the layer as on IF pictures where 15 – 17 protrusions through the filter can be observed per one filter. This could probably be attributed to the fact that on IF pictures we are able to detect all protrusions that came through the filter and the cell layer, but with SEM we can only detect protrusions that came all the way through the epithelial layer and came out at the apical side of the epithelium. In summary these results might indicate that gut commensals can enhance DC to send out more protrusions or that pathogens such as EPEC might even reduce the number of protrusions to dampen the sampling capacity of DC and in this way might also decrease the sampling efficiency of the gut content.

4.11. Summary

Bacterial diarrhoeas are still one of the major reasons of child mortality especially in developing countries. Among diarrhoeagenic bacteria are *E. coli* species including the enteropathogenic *E. coli* (EPEC) which is the main interest in this study. It is of utmost importance to unravel mechanisms by which EPEC cause disease and to devise possible ways to treat it. One of the promising ways to treat diarrhoeas is by using probiotic bacteria. During this project I assessed the influence of the probiotic *E. coli* strain Nissle 1917 (EcN) on different diarrhoeagenic *E. coli* species. *E. coli* Nissle 1917 was found to inhibit the growth of a number of diarrhoeagenic *E. coli* strains due to secretion of microcins.

In recent years it became apparent that even noninvasive enteropathogenic bacteria encounter immune cells already during very early stages of infection. That's happening due to the sampling of luminal contents by M cells and dendritic cells at the gastrointestinal barrier. In this work interactions of EPEC with immune cells namely monocytes and dendritic cells were studied. EPEC was found to decrease the viability of human monocytes after 5 hours of infection. This effect was found to be TTSS-dependent and EspD-dependent, however, the exact mechanisms remain unknown. Cells were dying by necrosis as was shown employing lactate dehydrogenase (LDH) assays and staining with anti-annexin V antibodies. Our current hypothesis based on the obtained data is that EspD directly causes a viability decrease due to its pore-forming abilities. However, these conclusions have to be investigated by future research.

Possible immunomodulatory effects of EPEC were studied using gene expression arrays, qRT-PCR, and mi-RNA arrays. Several proinflammatory cytokines were found to be upregulated in EPEC infected cells to a lesser extent than in the cells infected with probiotic *E. coli* strain. This effect was observed for both THP-1 cells and DC. These results are in agreement with the observation that EPEC doesn't cause severe inflammation in the gut - in contrast to *Salmonella* and *Shigella*. However, due to lack of time expression of not all of the affected genes were confirmed with alternative methods such as qRT-PCR. This should be

done in further experiments to come to final conclusions on the immunomodulatory abilities of EPEC. EPEC also blocks phagocytosis by THP-1 cells and DC, where the differences between EcN and EPEC uptake reaches 1000-fold.

To study certain aspects of host-pathogen interactions at the gastrointestinal barrier Transwell-based coculture model was used. This system includes Caco-2 epithelial cell line and primary human dendritic cells. Employing this model we observed that EcN supernatant induces the formation of protrusions by DC. This can be considered as one more beneficial effect of these probiotic bacteria as it presumably leads to more efficient sampling of luminal bacteria. However, it's currently not known what protein(s) is(are) responsible for the effect.

All in all it should be noted that interactions of EPEC and EcN with immune cells at the gastrointestinal barrier are quite complex and require more studies to gain a full understanding of processes that are taking place during the infection or colonization of the gut.

References

1. M. Abul-Milh, Y. Wu, B. Lau, C. A. Lingwood, and D. B. Foster. 2001. Induction of epithelial cell death including apoptosis by enteropathogenic *Escherichia coli* expressing bundle-forming pili. Infection and Immunity 69(12): 7356 – 7364.

2. A. Al-Shami, R. Spolski, J. Kelly, A. Keane-Myers, and W. J. Leonard. 2005. A role for TSLP in the development of inflammation in an asthma model. J. Exp. Med. 202: 829 – 839.

3. A. Altenhoefer, S. Oswald, U. Sonnenborn, C. Enders, J. Schulze, J. Hacker, T. A. Oelschlaeger. 2004. The probiotic *Escherichia coli* strain Nissle 1917 interferes with invasion of human intestinal epithelial cells by different enteroinvasive bacterial pathogens. FEMS immunology and med. microbiology 40(3): 223 – 9.

4. D. M. Anderson, and O. Schneewind. 1997. A mRNA signal for the type III secretion of Yop proteins by *Yersinia enterocolitica*. Science 278: 1140 – 1143.

5. B. Arribas, M. E. Rodríguez-Cabezas, D. Camuesco, M. Comalada, E. Bailón, P. Utrilla, A. Nieto, A. Concha, A. Zarzuelo, J. Gálvez. 2009. A probiotic strain of *Escherichia coli*, Nissle 1917, given orally exerts local and systemic anti-inflammatory effects in lipopolysaccharide-induced sepsis in mice. Br. J. Pharmacol. 157(6): 1024 – 33.

6. J. R. Arron, Y. Pewzner-Jung, M. C. Walsh, T. Kobayashi, and Y. Choi. 2002. Regulation of the subcellular localization of tumor necrosis factor receptor–associated factor (TRAF-2) by TRAF1 reveals mechanisms of TRAF2 signaling. J. Exp. Med. 196(7): 923 – 934.

7. D. Artis. 2008. Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut. Nature Reviews Immunology 8: 411 – 420.

8. Sh. Ashkenazi, Y. Amir, G. Dinari, T. Schonfeld, and M. Nitzan. 1983. Differential leukocyte count in acute gastroenteritis: an aid to early diagnosis. Clinical Pediatrics (Phila) 22: 356 – 358.

9. C. Atuma, V. Strugula, A. Allen, and L. Holm. 2001. The adherent gastrointestinal mucus gel layer: thickness and physical state in vivo. Am. J. Physiol. Gastrointest. Liver Physiol. 280: G922 – G929.

10. F. Backhed, R. E. Ley, J. L. Sonnenburg, D. A. Peterson, J. I. Gordon. 2005. Host-bacterial mutualism in the human intestine. Science 307: 1915 – 1920.

11. F. Baquero and F. Moreno. 1984. The microcins. FEMS Microbiology Letters 23: 117 – 124.

12. Chr. Beinke, Sv. Laarman, Cl. Wachter, H. Karch, L. Greune, and M. Alexander Schmidt. 1998. Diffusely Adhering *Escherichia coli* Strains Induce Attaching and Effacing Phenotypes and Secrete Homologs of Esp Proteins. Infection and immunity 66(2): 528 – 539.

13. C. N. Berger, V. F. Crepin, M. A. Jepson, A. Arbeola, and G. Frankel. 2009. The mechanisms used by enteropathogenic *Escherichia coli* to control filopodia dynamics. Cellular microbiology 11(2): 309 – 322.

14. F. Betis, P. Brest, V. Hofman, J. Guignot, M.-Fr. Bernet-Camard, B. Rossi, A. Servin, and P. Hofman. 2003. The Afa/Dr adhesins of diffusely adhering *Escherichia coli* stimulate interleukin-8 secretion, activate mitogen-activated protein kinases, and promote polymorphonuclear transepithelial migration in T84 polarized epithelial cells. Infection and Immunity 71(3): 1068 – 1074.

15. H. Birkedal-Hansen, W. G. I. Moore, M. K. Bodden, L J. Windsor, B. Birkedal-Hansen, A. DeCarlo, J. A. Engler. 1993. Matrix metalloproteinases: a review. Critical Reviews in Oral Biology and Medicine 4(2): 197 – 250.

16. H. von Boehmer. 2005. Mechanisms of suppression by suppressor T cells. Nature immunology 6(4): 338 – 344.

17. P. Bouillet, J. F. Purton, D. I. Godfrey, L. C. Zhang, L. Coultas, H. Puthalakath, M. Pellegrini, S. Cory, J. M. Adams, A. Strasser. 2002. BH3-only Bcl-2 family member Bim is required for apoptosis of autoreactive thymocytes. Nature 415(6874): 922 – 926.

18. P. Brest, Fr. Betis, N. Cuburu, E. Selva, M. Herrant, A. Servin, P. Auberger, and P. Hofman. 2004. Increased Rate of Apoptosis and Diminished Phagocytic Ability of Human Neutrophils Infected with Afa/Dr Diffusely Adhering *Escherichia coli* Strains. Infection and Immunity 72(10): 5741 – 5749.

19. J. Bryce, C. Boschi-Pinto, K. Shibuya, R. E. Black. 2005. WHO Child Health Epidemiology Reference Group. WHO estimates of the causes of death in children. Lancet 365: 1147 – 1152.

20. N. A. Buchmeier, and F. Heffron. 1991. Inhibition of macrophage phagosome-lysosome fusion by *Salmonella typhimurium*. Infect. Immun. 59: 2232 – 2238.

21. J. Celli, M. Olivier, and B. Br. Finlay. 2001. Enteropathogenic *Escherichia coli* mediates antiphagocytosis through the inhibition of PI 3-kinase-dependent pathways. EMBO J. 20(6): 1245 – 1258.

22. M. Chieppa, M. Rescigno, A.Y. Huang, and R.N. Germain. 2006. Dynamic imaging of dendritic cell extension into the small bowel lumen in response to epithelial cell TLR engagement. J. Exp. Med. 203: 2841 – 2852.

23. J. Cleary, L.-C. Lai, R. K. Shaw, A. Straatman-Iwanowska, M. S. Donnenberg, G. Frankel, and S. Knutton. 2004. Enteropathogenic *Escherichia coli* (EPEC) adhesion to intestinal epithelial cells: role of bundle-forming pili (BFP), EspA filaments and intimin. Microbiology 150: 527 – 538.

24. H. R. Conti, F. Shen, N. Nayyar, E. Stocum, J. N. Sun, M. J. Lindemann, A. W. Ho, J. Hoda Hai, J. J. Yu, J. W. Jung, Sc. G. Filler, P. Masso-Welch, M. Edgerton, and S. L. Gaffen. 2009. Th17 cells and IL-17 receptor signaling are essential for mucosal host defense against oral candidiasis. J. Exp. Med. 206(2): 299 – 311.

25. J. K. Crane, S. Majumdar, D. P. Pickhardt. 1999. Host cell death due to enteropathogenic *Escherichia coli* has features of apoptosis. Infect. Immun. 67: 2575 – 2584.

26. J. K. Crane, B. P. McNamara, and M. S. Donnenberg. 2001. Role of EspF in host cell death induced by enteropathogenic *Escherichia coli*. Cell Microbiol. 3: 197 – 211.

27. E. A. Creasey , R. M. Delahay, A. A. Bishop , R. K. Shaw , Br. Kenny, St. Knutton and G. Frankel. 2003. CesT is a bivalent enteropathogenic *Escherichia coli* chaperone required for translocation of both Tir and Map. Microbiology 47(1): 209 – 221.

28. E. A. Creasey, D. Friedberg, R. K. Shaw, T. Umanski, St. Knutton, I. Rosenshine and G. Frankel. 2003. CesAB is an enteropathogenic *Escherichia coli* chaperone for the type-III translocator proteins EspA and EspB . Microbiology 149: 3639-3647.

29. M. L. Cross, A. Ganner, D. Teilab, L. M. Fray. 2004. Patterns of cytokine induction by gram-positive and gram-negative probiotic bacteria. 2004. FEMS immunology and medical microbiology 42: 173 – 180.

30. S. J. Daniell, N. Takahashi, R. Wilson, D. Friedberg, I. Rosenshine, Fr. P. Booy, R. K. Shaw, St. Knutton, G. Frankel and Sh.-I. Aizawa. 2001. The filamentous type III secretion translocon of enteropathogenic *Escherichia coli*. Cell. Microbiology 3(12): 865 – 871.

31. P. Dean, M. Maresca, Br. Kenny. 2005. EPEC's weapons of mass subversion. Curr. Opin. Microbiology 8(1): 28 – 34.

32. M. A. Delgado, J. O. Sobiati, M. J. Chiuchiolo, R. N. Farias, and R. A. Salomon. 1999. *Escherichia coli* outer membrane protein TolC is involved in production of the peptide antibiotic microcin J25. J. Bacteriol. 181: 1968 – 1970.

33. M. Delgado, M. R. Rintoul, R. N. Farias, R. A. Salomon. 2001. *Escherichia coli* RNA polymerase is the target o the cyclopeptide antibiotic microcin J25. Journal of bacteriology 183(15): 4543 – 4550.

34. W. Deng, Y. Li, Ph. R. Hardwidge, E. A. Frey, R. A. Pfuetzner, S. Lee, S. Gruenheid, N. C. J. Strynakda, J. L. Puente, and B. Br. Finlay. 2005. Regulation of type III secretion

hierarchy of translocators and effectors in attaching and effacing bacterial pathogens. Infection and Immunity 73(4): 2135 – 2146.

35. D. Destomieux-Garzon, X. Thomas, M. Santamaria, Chr. Goulard, M. Barthelemy, B. Boscher, Y. Bessin, G. Molle, A.-M. Pons, L. Letellier, J. Peduzzi and S. Rebuffat. 2003. Microcin E492 antibacterial activity: evidence for a TonB-dependent inner membrane permeabilization on *Escherichia coli*. Molecular Microbiology 49(4): 1031 – 1041.

36. M. S. Donnenberg, S. B. Calderwood, A. Donohue-Rolfe, G. T. Keusch, and J. B. Kaper. 1990. Construction and analysis of TnphoA mutants of enteropathogenic *Escherichia coli* unable to invade HEp-2 cells. Infect. Immun. 58(6): 1565 – 1571.

37. M. S. Donnenberg, J. Yu, J. B. Kaper. 1993. A second chromosomal gene necessary for intimate attachment of enteropathogenic *Escherichia coli* to epithelial cells. J. Bacteriol. 175(15): 4670 – 80.

38. R. Devinney, I. Nisan, Sh. Ruschkowski, I. Rosenshine, and B. Br. Finlay. 2001. Tir tyrosine phosphorylation and pedestal formation are delayed in enteropathogenic *Escherichia coli* sepZ::TnphoA Mutant 30-5-1(3). Infect. Immun. 69(1): 559 – 563.

39. S. Duquesne, V. Petit, J. Peduzzi, S. Rebuffat. 2007. Structural and functional diversity of microcins, gene-encoded antibacterial peptides from *Enterobacteria*. J. Mol. Microbiol. Biotechnol. 13: 200 – 209.

40. S. J. Elliott, St. W. Hutcheson, M. S. Dubois, J. L. Mellies, L. A. Wainwright, M. Batchelor, G. Frankel, St. Knutton, and J. B. Kaper. 1999. Identification of CesT, a chaperone for the type III secretion of Tir in enteropathogenic *Escherichia coli*. Molecular Microbiology 33(6): 1176 – 1189.

41. S. J. Elliott, C. B. O'Connell, A. Koutsouris, C. Brinkley, M. S. Donnenberg, G. Hecht, and J. B. Kaper. 2002. A gene from the locus of enterocyte effacement that is required for enteropathogenic *Escherichia coli* to Increase tight-junction permeability encodes a chaperone for EspF. Infection and Immunity 70(5): 2271 – 77.

42. B. Br. Finlay and St. Falkow. 1997. Common themes in microbial pathogenicity revisited. Microbiology and molecular biology reviews 61(2): 136 – 169.

43. D. Fomenko, A. Veselovskii, I. Khmel. 2001. Regulation of microcin C51 operon expression: the role of global regulators of transcription. Research in Microbiology 152(5): 469 – 479.

44. G. Frankel, A. D. Phillips, L. R. Trabulsi, S. Knutton, G. Dougan, and S. Matthews. 2001. Intimin and the host cell—is it bound to end in Tir(s)? Trends Microbiol. 9: 214 – 218.

45. L. S. Gansheroff, M. R. Wachtel, A. D. O'Brien. 1999. Decreased adherence of enterohemorrhagic *Escherichia coli* to Hep-2 cells in the presence of antibodies that recognize the C-terminal region of intimin. Infection and Immunity 67(12): 6409 – 6417.

46. J. F. Gärtner, and M. A. Schmidt. 2004. LEE pathogenicity islands of atypical enteropathogenic *Escherichia coli* (ATEC): comparative analysis and transfer of function. Infect. Immun. 72(11): 6722 – 6728.

47. M. K. Gately, L. M. Renzetti, J. Magram, A. S. Stern, L. Adorini, U. Gubler, D. H. Presky. 1998. The interleukin-12/interleukin-12-receptor system: role in normal and pathologic immune responses. Annu. Rev. Immunol. 16: 495 – 521.

48. C. Gaggero, F. Moreno, M. Lavina. 1993. Genetic analysis of microcin H47 antibiotic system. J. Bacteriol. 175(17): 5420 – 5427.

49. N. S. Goncalves, C. Hale, G. Dougan, G. Frankel, and T. T. MacDonald. 2003. Binding of intimin from enteropathogenic *Escherichia coli* to lymphocytes and its functional consequences. Infect. Immun. 71: 2960 – 2965.

50. D. L. Goosney, J. Celli, Br. Kenny, and B. Br. Finlay. 1999. Enteropathogenic *Escherichia coli* inhibits phagocytosis. Infect. Immun. 67(2): 490 – 495.

51. U. Gophna, E. Z. Ron, and D. Graur. 2003. Bacterial type III secretion systems are ancient and evolved by multiple horizontal-transfer events. Gene 17: 151 – 163.

52. A. Grabig, D. Paclik, C. Guzy, A. Dankof, D. C. Baumgart, J. Erckenbrecht, B. Raupach, U. Sonnenborn, J. Eckert, R. R. Schumann, B. Wiedenmann, A. U. Dignass, and A. Sturm. 2006. *Escherichia coli* strain Nissle 1917 ameliorates experimental colitis via Toll-Like receptor 2- and Toll-Like receptor 4-dependent pathways. Infection and Immunity 74(7): 4075 – 4082.

53. M. de Grado, A. Gauthier, O. Steele-Mortimer, R. Devinney, B. B. Finlay. 1999. Identification of the intimin binding domain of Tir of enteropathogenic *Escherichia coli*. Cell. Microbiol. 1: 7–18.

54. J. Grodberg, and J. J. Dunn. 1998. *ompT* encodes the *Escherichia coli* outer membrane protease that cleaves T7 RNA polymerase during purification. J. Bacteriol. 170(3): 1245 – 1253.

55. L. Grozdanov, C. Raasch, J. Schulze, U. Sonnenborn, G. Gottschalk, J. Hacker, U. Dobrindt. 2004. Analysis of the genome structure of the nonpathogenic probiotic *Escherichia coli* strain Nissle 1917. J. Bacteriol. 186(16): 5432 – 41.

56. S. Gruenheid, I. Sekirov, N. A. Thomas, W. Deng, P. O'Donnell, D. Goode, Y. Li , E. A. Frey, N. F. Brown, P. Metalnikov, T. Pawson, K. Ashman and B. Br. Finlay. 2004. Identification and

characterization of NleA, a non-LEE-encoded type III translocated virulence factor of enterohaemorrhagic *Escherichia coli* O157:H7. Mol. Microbiol. 51(5): 1233 – 1249.

57. E. L. Hartland, M. Batchelor, R. M. Delahay, C. Hale, S. Matthews, G. Dougan, S. Knutton, I. Connerton, and G. Frankel. 1999. Binding of intimin from enteropathogenic *Escherichia coli* to Tir and to host cells. Mol. Microbiol. 32: 151 – 158.

58. C. L. Hattrup, and S. J. Gendler. 2008. Structure and function of the cell surface (tethered) mucins. Annu. Rev. Physiol 70: 431 – 457.

59. C. R. Hauck. 2002. Cell adhesion receptors – signaling capacity and exploitation by bacterial pathogens. Medical microbiology and immunology 191(2): 55 – 62.

60. S. Hegde, J. Pahne, and S. Smola-Hess. 2004. Novel immunosuppressive properties of interleukin-6 in dendritic cells: inhibition of NF-kB binding activity and CCR7 expression. The FASEB journal 18(12): 1439 – 41.

61. J. Henker, M. W. Laass, B. M. Blokhin, V. G. Maydannik, Y. K. Bolbot, M. Elze, C. Wolff, A. Schreiner, J. Schulze. 2008. Probiotic *Escherichia coli* Nissle 1917 versus placebo for treating diarrhea of greater than 4 days duration in infants and toddlers. Pediatr. Infect. Dis. J. 27(6): 494 – 499.

62. J. Henker, M. W. Laass, B. M. Blokhin, Y. K. Bolbot, V. G. Maydannik, M. Elze, C. Wolff, A. Schreiner, J. Schulze. 2007. The probiotic *Escherichia coli* strain Nissle 1917 (EcN) stops acute diarrhoea in infants and toddlers. Eur J Pediatr. 166(4): 311-8.

63. L. M. Higgins, G. Frankel, I. Connerton, N. S. Gonclaves, G. Dougan, and T. T. MacDonald. 1999. Role of bacterial intimin in colonic hyperplasia and inflammation. Science 285: 588 – 591.

64. L. V. Hooper, J. I. Gordon. 2001. Commensal host-bacterial relationships in the gut. Science 292: 1115 – 1118.

65. L. Hu, A. Umeda, S. Kondo and K. Amako. 1995. Typing of *Staphylococcus aureus* colonising human nasal carriers by pulsed-field gel electrophoresis. J. Med. Microbiol. 42: 127 – 132.

66. Q. Huang, D. Liu, P. Majewski, L. C. Schulte, J. M. Korn, R. A. Young, E. S. Lander, N. Hacohen. 2001. The plasticity of dendritic cell responses to pathogens and their components. Science 294: 870 – 875.

67. Chr. J. Hueck. 1998. Type III protein secretion systems in bacterial pathogens of animals and plants. Microbiol. Mol. Biol. Rev. 62(2): 379 – 433

68. T. Ide, S. Laarmann, L. Greune, H. Schillers, H. Oberleithner, and M. A. Schmidt**.** 2001. Characterization of translocation pores inserted into plasma membranes by type III-secreted Esp proteins of enteropathogenic *Escherichia coli*. Cell. Microbiol. 3**:** 669 – 679.

69. I. D. Iliev, G. Matteoli, and M. Rescigno. 2007. The yin and yang of intestinal epithelial cells in controlling dendritic cells function. JEM 204(10): 2253 – 2257.

70. A. Iwasaki, and B. L. Kelsall. 1999. Freshly isolated Peyer's patch, but not spleen, dendritic cells produce interleukin 10 and induce the differentiation of T helper type 2 cells. J. Exp. Med. 190: 229 –239.

71. M. H. Janga, Mi-Na Kweona, K. Iwatania, M. Yamamotoa, K. Teraharad, Ch.Sasakawaf, T. Suzukig, T. Nochid, Y. Yokotai, P. D. Rennertj, T. Hiroia, H. Tamagawaa, H. Iijimaa, J. Kunisawaa, Y. Yukid, and H. Kiyonoa. 2004. Intestinal villous M cells: An antigen entry site in the mucosal epithelium. PNAS 101(16): 6110 – 6115.

72. A. M. Johnson, R. S. Kaushik, N. J. Rotella, and Ph. R. Hardwidge. 2009. Enterotoxigenic *Escherichia coli* Modulates Host Intestinal Cell Membrane Asymmetry and Metabolic Activity. Infection and Immunity 77: 341 – 347.

73. W. Ju, A. Ogawa, J. Heyer, D. Nierhof, L. Yu, R. Kucherlapati, D. A. Shafritz, and E. P. Böttinger. 2006. Deletion of Smad2 in mouse liver reveals novel functions in hepatocyte growth and differentiation. Mol. Cell. Biol. 26(2): 654 – 667.

74. N. Kamada, K. Maeda, N. Inoue, T. Hisamatsu, S. Okamoto, K. S. Hong, T. Yamada, N. Watanabe, K. Tsuchimoto, H. Ogata, T. Hibi. 2008. Nonpathogenic *Escherichia coli* strain Nissle 1917 inhibits signal transduction in intestinal epithelial cells. Infection and Immunity 76(1): 214 – 220.

75. Kr. J. Kanack, J. A. Crawford, I. Tatsuno, M. A. Karmali and J. B. Kaper. 2005. SepZ/EspZ is secreted and translocated into HeLa cells by the enteropathogenic *Escherichia coli* type III secretion system. Infection and Immunity 73(7): 4327 – 4337.

76. J. B. Kaper, J. P. Nataro, H. L. Mobley. 2004. Pathogenic *Escherichia coli*. Nat. Rev. Microbiol. 2: 123-40.

77. B. Kartmann, D. Roth. 2001. Novel roles for mammalian septins: from vesicle trafficking to oncogenesis. J. Cell. Sci. 114: 839 – 844.

78. G. Kelly, S. Prasannan, S. Daniell, K. Flemming, G. Frankel, G. Dougan. 1999. Intimin from enteropathogenic *E. coli* belongs to a new family of bacterial adhesion molecules containing C-type lectin- and tandem immunoglobulin-like domains. Nat. Struct. Biol. 6: 313 – 318.

79. Br. Kenny, R. DeVinney, M. Stein, D. J. Reinscheid, E. A. Frey, and B. B. Finlay. 1997. Enteropathogenic *E. coli* (EPEC) transfers its receptor for intimate adherence into mammalian cells. Cell 91: 511 – 520.

80. Br. Kenny. 1999. Phosphorylation of tyrosine 474 of the enteropathogenic *Escherichia coli* (EPEC) Tir receptor molecule is essential for actin nucleating activity and is preceded by additional host modifications. Mol. Microbiol. 31: 1229 – 1241.

81. Br. Kenny, S. Ellis, A. D. Leard, J. Warawa, H. Mellor, and M. A. Jepson. 2002. Co-ordinate regulation of distinct host cell signalling pathways by multifunctional enteropathogenic *Escherichia coli* effector molecules. Mol. Microbiol. 44: 1095 – 1107.

82. Br. Kenny, and M. Jepson. 2000. Targeting of an enteropathogenic *E. coli* (EPEC) effector protein to host mitochondria. Cell. Microbiol. 2: 579 – 590.

83. M. A. S. Khan, J. Kang, and Th. S. Steiner. 2004. Enteroaggregative *Escherichia coli* flagellin-induced interleukin-8 secretion requires Toll-like receptor 5-dependent p38 MAP kinase activation. Immunology 112: 651 – 660.

84. J. Kim, A. Thanabalasuriar, T. Chaworth-Musters, J. Chr. Fromme, E. A. Frey, P. I. Lario, P. Metalnikov, K. Rizg, N. A. Thomas, S. F. Lee, E. L. Hartland, Ph. R. Hardwidge, T. Pawson, N. C. Strynadka, B. Br. Finlay, R. Schekman, and S. Gruenheid. 2007. The bacterial virulence factor NleA inhibits cellular protein secretion by disrupting mammalian COPII function. Cell Host and Microbe 2(3): 160 – 171.

85. St. Knutton, M. M. Baldini, J. B. Kaper, and A. S. McNeish. 1987. Role of plasmid-encoded adherence factors in adhesion of enteropathogenic *Escherichia coli* to HEp-2 cells. Infect. Immun. 55: 78 – 85.

86. St. Knutton, I. Rosenshine, M. J. Pallen, I. Nisan, B. C. Neves, Chr. Bain, C. Wolff, G. Dougan, and G. Frankel. 1998. A novel EspA-associated surface organelle of enteropathogenic *Escherichia coli* involved in protein translocation into epithelial cells. EMBO J. 17(8): 2166 – 2176.

87. T. Korn, E. Bettelli, M. Oukka, V. K. Kuchroo. 2009. IL-17 and Th17 Cells. Annu. Rev. Immunol. 27: 485 – 517.

88. J.-P. Kraehenbuhl, and M. R. Neutra. 2000. Epithelial M cells: Differentiation and Function. Annu. Rev. Cell Dev. Biol. 16: 301 – 32.

89. N. E. Kurepina, E. I. Basyuk, A. Z. Metlitskaya, Dm. A. Zaitsev, I. A. Khmel. 1993. Cloning and mapping of the genetic determinants for microcin C51 production and immunity. Mol. Gen. Genet. 241: 700 – 706.

90. L. C. Lai, L. A. Wainwright, K. D. Stone and M. S. Donnenberg. 1997. A third secreted protein that is encoded by the enteropathogenic *Escherichia coli* pathogenicity island is required for transduction of signals and for attaching and effacing activities in host cells. Infect. Immun. 65(6): 2211 – 2217.

91. R. Lan, M. Ch. Alles, K. Donohoe, M. B. Martinez, and P. R. Reeves. 2004. Molecular evolutionary relationships of enteroinvasive *Escherichia coli* and *Shigella* spp. Infection and Immunity 72(9): 5080 – 5088.

92. M. A. Lasaro, J. F. Rodrigues, C. Mathias-Santos, B. E. C. Guth, A. Balan, M. E. Sbrogio-Almeida, and L. C. S. Ferreira. 2008. Genetic diversity of heat-labile toxin expressed by enterotoxigenic *Escherichia coli* strains isolated from humans. Journal of Bacteriology 190(7): 2400 – 2410.

93. M. P. Leatham, S. Banerjee, S. M. Autieri, R. Mercado-Lubo, T. Conway, P. S. Cohen. 2009. Precolonized human commensal *Escherichia coli* strains serve as a barrier to *E. coli* O157:H7 growth in the streptomycin-treated mouse intestine. Infect Immun. 77(7): 2876 – 86.

94. J. D. Li, W. Feng, M. Gallup, J. H. Kim, J. Gum, Y. Kim, and C. Basbaum. 1998. Activation of NF-kappaB via a Src-dependent Ras-MAPK-pp90rsk pathway is required for *Pseudomonas aeruginosa*-induced mucin overproduction in epithelial cells. PNAS 95: 5718 – 5723.

95. S. K. Linden, T. H. Florin, and M. A. McGuckin. 2008. Mucin dynamics in intestinal bacterial infection. PLoS. ONE. 3: e3952.

96. M. A. Lindsay. 2008. MicroRNAs and the immune response. Trends in Immunology 29 (7): 343 – 351.

97. P. Ling, M. K. Gately, U. Gubler, A. S. Stern, P. Lin, K. Hollfelder, Ch. Su, Y.-Ch. E. Pan, and J. Hakimi. 1995. Human IL-12 p40 homodimer binds to the IL-12 receptor but doesn't mediate biologic activity. The Journal of immunology 154(1): 116 – 27.

98. Y.-J. Liu, V. Soumelis, N. Watanabe, T. Ito, Y.-H. Wang, R. de Waal Malefyt, M. Omori, B. Zhou, and S. F. Ziegler. 2007. TSLP: an epithelial cells cytokine that regulates T cell differentiation by conditioning dendritic cells maturation. Annual reviews immunology 25: 193 – 219.

99. A. J. Macpherson and Th. Uhr. 2004. Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria. Science 303: 1662 – 1665.

100. A. J. Macpherson and Th. Uhr. 2004. Compartmentalization of the mucosal immune responses to commensal intestinal bacteria. Ann. N.Y. Acad. Sci. 1029: 36 – 43.

101. O. Marches, V. Covarelli, S. Dahan, C. Cougoule, P. Bhatta, G. Frankel, and E. Caron. 2008. EspJ of enteropathogenic and enterohemorrhagic *Escherichia coli* inhibits opsono-phagocytosis. Cell. Microbiol. 10(5): 1104 – 1115.

102. Th. C. Marlovits, T. Kubori, A. Sukhan, D. R. Thomas, J. E. Galán, V. M. Unger. 2004. Structural insights into the assembly of the type III secretion needle complex. Science 306(5698): 1040 – 1042.

103. T. K. McDaniel, K. G. Jarvis, M. S. Donnenberg, and J. B. Kaper. 1995. A genetic locus of enterocyte effacement conserved among diverse enterobacterial pathogens. PNAS 92(5): 1664 – 1668.

104. B. P. McNamara, A. Koutsouris, C. B. O'Connell, J. P. Nougayrede, M. S. Donnenberg, and G. Hecht. 2001. Translocated EspF protein from enteropathogenic *Escherichia coli* disrupts host intestinal barrier function. J. Clin. Investig. 107: 621 – 629.

105. A. Menendez and B. B. Finlay. 2007. Defensins in the immunology of bacterial infections. Current opinion in immunology 19: 385 – 391.

106. A. Z. Metlitskaya, G. S. Katrukha, A. S. Shashkov, Dm. A. Zaitsev, Ts. A. Egorov, I. A. Khmel. 1995. Structure of microcin C51, a new antibiotic with a broad spectrum of activity. FEBS Letters 357: 235 – 238.

107. S. Michgehl, G. Heusipp, L. Greune, Chr. Rüter and M. A. Schmidt. 2006. Esp-independent functional integration of the translocated intimin receptor (Tir) of enteropathogenic *Escherichia coli* (EPEC) into host cell membranes. Cellular Microbiology 8(4): 625 – 633.

108. T. Michiels, and G. R. Cornelis. 1991. Secretion of hybrid proteins by the *Yersinia* Yop export system. J. Bacteriol. 173: 1677 – 1685.

109. H. Miller, J. Zhang, Rh. KuoLee, G. B. Patel, and W. Chen. 2007. Intestinal M cells: The fallible sentinels? World J. Gastroenterol. 13(10): 1477 – 1486.

110. M. Moendel, B. O. Schroeder, K. Zimmermann, H. Huber, S. Nuding, J. Beisner, K. Fellermann, E. F. Stange, J. Wehkamp J. 2009. Probiotic *E. coli* treatment mediates antimicrobial human beta-defensin synthesis and fecal excretion in humans. Mucosal Immunol. 2(2): 166 – 72.

111. J. A. Mohamed, D. B. Huang, Zh.-D. Jiang, H. L. DuPont, J. P. Nataro, J. Belkind-Gerson, and P. C. Okhuysen. 2007. Association of putative enteroaggregative *Escherichia coli* virulence genes and biofilm production in isolates from travelers to developing countries. Journal of Clinical Microbiology 45(1): 121 – 126.

112. J. R. Mora, M. Iwata, B. Eksteen, S. Y. Song, T. Junt, B. Senman, K. L. Otipoby, A. Yokota, H. Takeuchi, and P. Ricciardi-Castagnoli. 2006. Generation of gut-homing IgA-secreting B cells by intestinal dendritic cells. Science 314: 1157 – 1160.

113. S. Mostowy, T. N. Tham, A. Danckaert, St. Guadagnini, St. Boisson-Dupuis, J. Pizarro-Cerdá, and Pascale Cossart. 2009. Septins regulate bacterial entry into host cells. PLoS ONE 4(1): e4196.

114. D. Müller, P. Hagedorn, S. Brast, G. Heusipp, M. Bielaszewska, A. W. Friedrich, H. Karch, and M. A. Schmidt. 2002. Rapid identification and differentiation of clinical isolates of enteropathogenic *Escherichia coli* (EPEC), atypical EPEC, and Shiga toxin-producing *Escherichia coli* by a one-step multiplex PCR method. Journal of Clinical Microbiology 33(6): 1176 – 1189.

115. D. Müller, L. Greune, G. Heusipp, H. Karch, A. Fruth, H. Tschäpe, and M. A. Schmidt. 2007. Identification of unconventional intestinal pathogenic *Escherichia coli* expressing intermediate virulence factor profiles with a novel single step multiplex PCR. Appl. Environ. Microbiol. 73: 3380 – 3390.

116. D. Müller, I. Benz, A. Liebchen, I. Gallitz, H. Karch, and M. A. Schmidt. 2009. Comparative analysis of the locus of enterocyte effacement and its flanking regions. Infect. Immun. 77: 3501 – 3513.

117. R. Mundy, L. Petrovska, K. Smollett, N. Simpson, R. K. Wilson, J. Yu, X. Tu, I. Rosenshine, S. Clare, G. Dougan, and G. Frankel. 2004. Identification of a novel *Citrobacter rodentium* type III secreted protein, EspI, and roles of this and other secreted proteins in infection. Infection and Immunity 72(4): 2288 – 2302.

118. J. P. Nataro, J. B. Kaper. 1998. Diarrhoeagenic *Escherichia coli.* Clin. Microbiol. Rev. 11: 142 – 201.

119. R. N. Nguyen, L. S. Taylor, M. Tauschek, and R. M. Robins-Browne. 2006. Atypical enteropathogenic *Escherichia coli* infection and prolonged diarrhea in children. Emerging Infectious Diseases 12(4): 597 – 603.

120. C. Nicoletti. 2000. Unsolved mysteries of intestinal M cells. Gut 47: 735 – 739.

121. A. Nissle. 1925. Weiteres über Grundlagen und Praxis der Mutaflorbehandlung. DMW 4: 1809 –1813.

122. Th. J. Ochoa, Fr. Barletta, C. Contreras, and E. Mercado. 2008. New insights into the epidemiology of enteropathogenic *Escherichia coli* infection. Trans. R. Soc. Trop. Med. Hyg. 102(9): 852 – 856.

123. C. B. O'Connell, E. A. Creasey, St. Knutton, S. Elliott, L. J. Crowther, W. Luo, M. J. Albert, J. B. Kaper, G. Frankel, and M. S. Donnenberg. 2004. SepL, a protein required for enteropathogenic *Escherichia coli* type III translocation, interacts with secretion component SepD. Mol Microbiol. 52(6): 1613 – 1625.

124. Y. Ogura, T. Ooka, A. Whale, J. Garmendia, L. Beutin, Sh. Tennant, Gl. Krause, St. Morabito, I. Chinen, T. Tobe, H. Abe, R. Tozzoli, A. Caprioli, M. Rivas, R. Robins-Browne, T. Hayashi, and G. Frankel. 2007. TccP2 of O157:H7 and Non-O157 enterohemorrhagic *Escherichia coli* (EHEC): challenging the dogma of EHEC-induced actin polymerization. Infection and Immunity 75(2): 604 – 612.

125. R. L. Owen and A. L. Jones. 1974. Epithelial cell specialisation within Peyer's patches: an ultrastructural study of human intestinal lymphoid follicles. Gastroenterology 66: 189 –203.

126. M. K. Oyoshi, R. Barthel, and E. N. Tsitsikov. 2007. TRAF1 regulates recruitment of lymphocytes and, to a lesser extent, neutrophils, myeloid dendritic cells and monocytes to the lung airways following lipopolysaccharide inhalation. Immunology 120(3): 303 – 314.

127. J. Papavassiliou. 1959. Production of colicines in Simmons's citrate agar. Nature 184: 1339 –1340.

128. J. Papavassiliou. 1961. Biological characteristics of colicine X. Nature 190: 110.

129. S. I. Patzer, M. R. Baquero, D. Bravo, F. Moreno, and Kl. Hantke. 2003. The colicin G, H and X determinants encode microcins M and H47, which might utilize the catecholate siderophore receptors FepA, Cir, Fiu and IroN. Microbiology 149: 2557 – 2570.

130. St. Pils, T. Schmitter, Fl. Neske and Chr. Hauck. 2006. Quantification of bacterial invasion into adherent cells by flow cytometry. Journal of Microbiological Methods 65(2): 301 – 310.

131. M. F. Pomares, P. A. Vincent, R. N. Farias, and R. A. Salomon. 2008. Characterization of the *relA1* mutation and a comparison of *relA1* with new *relA* null alleles in *Escherichia coli*. Journal of bacteriology 190(12): 4328 – 4334.

132. A.-M. Pons, N. Zorn, D. Vignon, Fr. Delalande, A. V. Dorsselaer, and G. Cottenceau. 2002. Microcin E492 is an unmodified peptide related in structure to colicin V. Antimicrob. Agents Chemother. 46(1): 229 – 230.

133. A. P. Pugsley, F. Moreno, and V. DeLorenzos. 1986. Microcin-E492-insensitive mutants of *Escherichia coli* K12. Journal of General Microbiology 132: 3253 – 3259.

134. F. Qadri, A.-M. Svennerholm, A. S. G. Faruque, and R. B. Sack. 2005. Enterotoxigenic *Escherichia coli* in developing countries: epidemiology, microbiology, clinical features, treatment, and prevention. Clin. Microbiology Rev. 18(3): 465 – 483.

135. R. P. Rabinowitz, L.-Ch. Lai, K. Jarvis, T. K. McDaniel, J. B. Kaper, K. D. Stone and M. S. Donnenberg. 1996. Attaching and effacing of host cells by enteropathogenic *Escherichia coli* in the absence of detectable tyrosine kinase mediated signal transduction. Microbial Pathogenesis 21(3): 157 – 171.

136. S. Rakoff-Nahoum, J. Paglino, F. Eslami-Varzaneh, S. Edberg, and R. Medzhitov. 2004. Recognition of commensal microflora by Toll-like receptors is required for intestinal homeostasis. Cell 118: 229 – 241.

137. R. Reissbrodt, W. P. Hammes, F. dal Bello, R. Prager, A. Fruth, Kl. Hantke, A. Rakin, M. Starcic-Erjavec, P. H. Williams. 2009. Inhibition of growth of Shiga toxin-producing *Escherichia coli* by nonpathogenic *Escherichia coli*. FEMS Microbiol Lett. 290(1): 62 – 9.

138. M. Rescigno, M. Urbano, B. Valzasina, M. Francolini, G. Rotta, R. Bonasio, Fr. Granucci, J.-P. Kraehenbuh, and P. Ricciardi-Castagnoli. 2001. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. Nat. Immunol. 2: 361 – 367.

139. M. Rimoldi, M. Chieppa, V. Salucci, F. Avogadri, A. Sonzogni, G.M. Sampietro, A. Nespoli, G. Viale, P. Allavena, and M. Rescigno. 2005. Intestinal immune homeostasis is regulated by the crosstalk between epithelial cells and dendritic cells. Nat. Immunol. 6: 507 – 514.

140. E. Rodriguez, and M. Lavina. 1998. Genetic analysis of microcin H47 immunity. Can. J. Microbiol. 44: 692 – 697.

141. E. Rodriguez, and M. Lavina. 2003. The proton channel is the minimal structure of ATP synthase necessary and sufficient for microcin H47 antibiotic action. Antimicrob. Agents Chemother. 47(1): 181 – 187.

142. A. Rodriguez, E. Vigorito, S. Clare, M. V. Warren, Ph. Couttet, D. R. Soond, St. van Dongen, R. J. Grocock, P. P. Das, E. A. Miska, D. Vetrie, Kl. Okkenhaug, A. J. Enright, G. Dougan, M. Turner, A. Bradley. 2007. Requirement of bic/microRNA-155 for normal immune function. Science 316(5824): 608 – 611.

143. P. M. Rood, J. Calafat, A. E. von dem Borne, W. R. Gerritsen, C. E. van der Schoot. 2000. Immortalisation of human bone marrow endothelial cells: characterization of new cell lines. Eur. J. Clin. Invest. 30: 618 – 629.

144. R. Rosqvist, I. Bolin, and H. Wolf-Watz. 1998. Inhibition of phagocytosis in *Yersinia pseudotuberculosis*: a virulence plasmid-encoded ability involving the Yop2b protein. Infect. Immun. 56: 2139 – 2143.

145. K. Roy, G. M. Hilliard, D. J. Hamilton, J. Luo, M. M. Ostmann, and J. M. Fleckenstein. 2009. Enterotoxigenic *Escherichia coli* EtpA mediates adhesion between flagella and host cells. Nature 457(7229): 594 – 598.

146. M.-H. Ruchaud-Sparagano, M. Maresca, B. Kenny. 2007. Enteropathogenic *Escherichia coli* (EPEC) inactivate innate immune responses prior to compromising epithelial barrier function. Cell. Microbiol. 9(8): 1909 – 1921.

147. A. Samba-Louaka, J.-Ph. Nougayrède, Cl. Watrin, E. Oswald, and Fr. Taieb. 2009. The EPEC effector Cif induces delayed apoptosis in epithelial cells. Infect. Immun. Electronic publication ahead of print.

148. Ph. J. Sansonetti. 2001. Phagocytosis of bacterial pathogens: implications in the host response. Semin. Immunol. 13: 381 – 390.

149. Ph. J. Sansonetti. 2004. War and peace at mucosal surfaces. Nat. Rev. Immunology 4: 953 – 964.

150. A. M. Sato, M. Hashiguchi, E. Toda, A. Iwasaki, S. Hachimura, and S. Kaminogawa. 2003. CD11b+ Peyer's patch dendritic cells secrete IL-6 and induce IgA secretion from naive B cells. J. Immunol. 171: 3684 – 3690.

151. E. Schütz. 1989. The treatment of intestinal diseases with Mutaflor. A multicenter retrospective study. Fortschr. Med. 107(28): 599 – 602.

152. T. Sekiya, Sh. Adachi, K. Kohu, T. Yamada, O. Higuchi, Y. Furukawa, Y. Nakamura, Ts. Nakamura, K. Tashiro, S. Kuhara, S. Ohwada, and T. Akiyama. 2003. Identification of BMP and activin membrane-bound inhibitor (BAMBI), an inhibitor of transforming growth factor-beta signaling, as a target of the beta-catenin pathway in colorectal tumor cells. J Biol Chem 279 (8): 6840 – 6.

153. A. L. Servin. 2005. Pathogenesis of Afa/Dr Diffusely Adhering *Escherichia coli*. Clin. Microbiol. Rev. 18(2): 264 – 292.

154. R. K. Shaw, J. Cleary, M. S. Murphy. 2005. Interaction of enteropathogenic *Escherichia coli* with human intestinal mucosa: role of effector proteins in brush border remodeling and formation of attaching and effacing lesions. Infection and Immunity 73(2): 1243 – 1251.

155. J. F. Sinclair, and A. D. O'Brien. 2002. Cell surface-localized nucleolin is a eukaryotic receptor for the adhesin intimin-gamma of enterohemorrhagic *Escherichia coli* O157:H7. J. Biol. Chem. 279: 2876 – 2885.

156. H. Smith. 1968. Biochemical challenge of microbial pathogenicity. Bacteriol. Rev. 32: 164 – 184.

157. G. E. Soto and Sc. J. Hultgren. 1999. Bacterial adhesins: common themes and variations in architecture and assembly. J. Bacteriol. 181(4): 1059 – 1071.

158. J. L. Sonnenburg, J. Xu, D. D. Leip, C. H. Chen, B. P. Westover, J. Weatherford, J. D. Buhler, and J. I. Gordon. 2005. Glycan foraging in vivo by an intestine-adapted bacterial symbiont. Science 307: 1955 – 1959.

159. V. Soumelis, P. A. Reche, H. Kanzler, W. Yuan, G. Edward, B. Homey, M. Gilliet, S. Ho, S. Antonenko, and A. Lauerma. 2002. Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. Nat. Immunol. 3: 673 – 680.

160. R. M. Steinman. 1991. The dendritic cells and their role in immunogenicity system. Ann. Rev. Immunology 9: 271 – 296.

161. F. W. Studier, and B. A. Moffatt. 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. JMB 189: 113-130.

162. K. D. Taganov, M. P. Boldin, K.-J. Chang, and D. Baltimore. 2006. NF-κB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. PNAS 103(33): 12481 – 12486.

163. K. A. Taylor, C. B. O'Connell, P. W. Luther, and M. S. Donnenberg. 1998. The EspB protein of enteropathogenic *Escherichia coli* is targeted to the cytoplasm of infected HeLa cells. Infect. Immun. 66: 5501 – 5507.

164. K. A. Taylor, P. W. Luther, and M. S. Donnenberg. 1999. Expression of the EspB protein of enteropathogenic *Escherichia coli* within HeLa cells affects stress fibers and cellular morphology. Infect. Immun. 67: 120 – 125.

165. C. M. Thorpe. 2004. Shiga-toxin producing *Escherichia coli* infection. Clin. Infect. Dis. 38: 1298 – 1303.

166. L. G. Tilney, O. S. Harb, P. S. Connelly, C. G. Robinson and Cr. R. Roy. 2001. How the parasitic bacterium *Legionella pneumophila* modifies its phagosome and transforms it into rough ER: implications for conversion of plasma membrane to the ER membrane. JCS 114: 4637 – 4650.

167. T. Tobe, T. Hayashi, Ch.-G. Han, G. K. Schoolnik, E. Ohtsubo, and Ch. Sasakawa. 1999. Complete DNA sequence and structural analysis of the enteropathogenic *Escherichia coli* adherence factor plasmid. Infection and Immunity 67(10): 5455 – 5462.

168. A. G. Torres, X. Zhou, and J. B. Kaper. 2005. Adherence of diarrheagenic *Escherichia coli* strains to epithelial cells. Infection and Immunity 73(1): 18 – 29.

169. L. R. Trabulsi, R. Keller, and T. A. Tardelli Gomes. 2002. Typical and atypical enteropathogenic *Escherichia coli*. Emerging Infectious Diseases. 8(5): 1851 – 1855.

170. W. S. Trimble. 1999. Septins: a highly conserved family of membrane-associated GTPases with functions in cell division and beyond. J. Membr. Biol. 169: 75 – 81.

171. M. Trujillo, E. Rodriguez, and M. Lavina. ATP synthase is necessary for microcin H47 antibiotic action. 2001. Antimicrobial Agents and Chemotherapy 45(11): 3128 – 3131.

172. E. N. Tsitsikov, D. Laouini, I. F. Dunn, T. Y. Sannikova, L. Davidson, F. W. Alt, R. S. Geha. TRAF1 is a negative regulator of TNF signaling. 2001. Enhanced TNF signaling in TRAF1-deficient mice. Immunity 15: 647 – 57.

173. X. Tu, I. Nisan, C. Yona, E. Hanski, and I. Rosenshine. 2003. EspH, a new cytoskeleton-modulating effector of enterohaemorrhagic and enteropathogenic Escherichia coli. Mol. Microbiol. 47: 595 – 606.

174. S. N. Ukena, A. Singh, U. Dringenberg, R. Engelhardt, U. Seidler, W. Hansen, A. Bleich, D. Bruder, A. Franzke, G. Rogler, S. Suerbaum, J. Buer, F. Gunzer, and A. M. Westendorf. 2007. Probiotic *Escherichia coli* Nissle 1917 inhibits leaky gut by enhancing mucosal integrity. PLoS One. 2(12): e1308.

175. M. Valdebenito, A. L. Crumbliss, G. Winkelmann, and Kl. Hantke. 2006. Environmental factors influence the production of enterobactin, salmochelin, aerobactin, and yersiniabactin in *Escherichia coli* strain Nissle 1917. Int. J. Med. Microbiol. 296(8): 513 – 20.

176. R. E. Vance, R. R. Isberg, and D. A. Portnoy. 2009. Patterns of pathogenesis: discrimination of pathogenic and nonpathogenic microbes by the innate immune system. Cell host and microbe 6: 10 – 21.

177. G. Vassiliadis, D. Destomieux-Garzon, C. Lombard, S. Rebuffat, and J. Peduzzi. 2009. Isolation and characterization of two members of the siderophore-microcin family, microcins M and H47. Antimicrob. Agents Chemother.: online publication ahead of print.

178. L. A. Wainwright and J. B. Kaper. 2002. EspB and EspD require a specific chaperone for proper secretion from enteropathogenic *Escherichia coli*. Molecular Microbiology 27(6): 1247 – 1260.

179. Kr. Walther, M. K. Diril, N. Jung, and V. Haucke. 2003. Functional dissection of the interactions of stonin 2 with the adaptor complex AP-2 and synaptotagmin. PNAS 101(4): 964 – 969.

180. J. Warawa, B. Br. Finlay, and Br. Kenny. 1999. Type III secretion-dependent hemolytic activity of enteropathogenic *Escherichia coli*. Infect. Immun. 67(10): 5538 – 5540.

181. D. J. J. Waugh and C. Wilson. 2008. The Interleukin-8 Pathway in Cancer. Clinical Cancer Research 14: 6735 – 6741.

182. J. Wehkamp, J. Harder, K. Wehkamp, M. B. von Wehkamp, M. Schlee, C. Enders, U. Sonnenborn, S. Nuding, S. Bengmark, K. Fellermann, J. M. Schroeder, E. F. Stange. 2004. NF-kappaB- and AP-1-mediated induction of human beta defensin-2 in intestinal epithelial cells by *Escherichia coli* Nissle 1917: a novel effect of a probiotic bacterium. Infect. Immun. 72(10): 5750 – 5758.

183. C. Winstanley, C. A. Hart. 2001. Type III secretion systems and pathogenicity islands. J. Med. Microbiol. 50(2): 116 – 26.

184. Br. W. Wren. 2000. Microbial genome analysis: insights into virulence, host adaptation and evolution. Nature reviews genetics 1(1): 30 – 39.

185. J. M. Yoo, M. Omori, D. Gyarmati, B. Zhou, T. Aye, A. Brewer, M.R. Comeau, D.J. Campbell, and S.F. Ziegler. 2005. Spontaneous atopic dermatitis in mice expressing an inducible thymic stromal lymphopoietin transgene specifically in the skin. J. Exp. Med. 202: 541 – 549.

186. J. Yuzhenkova, M. Delgado, S. Nechaev, D. Savalia, V. Epstein, I. Artsimovitch, R. A. Mooney, R. Landick, R.N. Farias, R. Salomon, and K. Severinov. 2002. Mutations of bacterial RNA polymerase leading to resistance to microcin J25. J. Biol. Chem. 277: 50867 – 50875.

187. B. Zhou, M. R. Comeau, T. De Smedt, H. D. Liggitt, M. E. Dahl, D. B. Lewis, D. Gyarmati, T. Aye, D. J. Campbell, and S. F. Ziegler. 2005. Thymic stromal lymphopoietin as a key initiator of allergic airway inflammation in mice. Nat. Immunol. 6: 1047 – 1053.

188. E. G. Zoetendal, M. Rajilic-Stojanovic, and W. M. de Vos. 2008. High-throughput diversity and functionality analysis of the gastrointestinal tract microbiota. Gut 57: 1605 – 1615.

189. D. V. Zurawski, K. L. Mumy, L. Badea, J. A. Prentice, E. L. Hartland, B. A. McCormick, A. T. Maurelli. 2008. The NleE/OspZ family of effector proteins is required for polymorphonuclear transepithelial migration, a characteristic shared by enteropathogenic *Escherichia coli* and *Shigella flexneri* infections. Infect. Immun. 76: 369 – 379.

190. A. A. Zyrek, Chr. Cichon, S. Helms, C. Enders, U. Sonnenborn, and M. A. Schmidt. 2006. Molecular mechanisms underlying the probiotic effects of *Escherichia coli* Nissle 1917 involve ZO-2 and PKCζ redistribution resulting in tight junction and epithelial barrier repair. Cellular Microbiology 9(3): 804 – 816.

Lebenslauf

Vorname, Name: Nadzeya Kramko

geboren am: 31.08.1984. in Minsk, Weißrussland;

Familienstand: ledig

Name des Vaters: Yauhen Kramko

Name der Mutter: Liudmila Kramko, geb. Bulanovich

Schulbildung: Grundschule: von 1990 bis 2001 in School No 168, Minsk, Weißrussland

Hochschulreife (Abitur): am 2001 in Minsk, Weißrussland

Studium: Weißrussische Staatsuniversität: von 2001 bis 2006

Promotionsstudiengang: 2007 – 2009

Prüfungen: Diplom im Fach Biologie am Juni 2006, Weißrussische Staatsuniversität

Tätigkeiten: wissenschaftliche Mitarbeiterin von August 2006 bis Dezember 2006, molekular Biologie Labor, Weißrussische Staatsuniversität, Kurchatov str. 5, Minsk, Weißrussland, Arbeitgeber Prof. Dr. Evtushenkov.

Beginn der Dissertation: Januar 2007, Infektiologie Institut, Von-Esmarch str. 56, Betreuer Prof. Dr. M. A. Schmidt.

.....

(Unterschrift)

Acknowledgements

I would like to thank my scientific advisor Prof. Dr. Alexander Schmidt for always finding time for me, for allowing me to carry out my ideas and giving me good advice in case of problems.

My working group for the fruitful discussions in the institute and for the good time outside the labs. Lilo for excellent electron microscopy pictures and for introducing me into the cell culture techniques. Cristoph for invaluable contribution in microarrays data analysis and for advice on other experiments. Inga for keeping strains collection and chemicals together with corresponding databases in truly German order which helped a lot. Verena for helping with the coculture model.

My second examiner Prof. Dr. Volker Gerke and third examiner Prof. Dr. Berenike Maier for great discussions and for agreeing to be members of my triumvirat.

Graduate school GRK1409 'Molecular interactions of pathogens with biotic and abiotic surfaces' for giving me an excellent opportunities for research to accomplish my PhD project. I would like to particularly thank coordinator of the graduate school Dr. Waltraud Walz-Schmidt for always being helpful and for lessons of German language.

DFG for funding my 3 years PhD studentship, including 5 weeks lab exchange and intraschool social events.

Dr. Maria Rescigno from European Institute of Oncology in Milano for giving me an opportunity to master useful techniques for my work and all lab members from for being helpful.

My family for supporting me from 2000 km distance.

All my friends for support and for making my stay in Münster fun and unforgettable.

Ivan for being around in stress and in joy and for reading and making useful comments on 150 pages of my thesis without complaint.