Institut Pasteur
Unité de Pathogénie Microbienne Moléculaire

THÈSE

Présentée à

L'Université Pierre et Marie Curie

Ecole doctorale : ED515 « Complexité du Vivant »

"The role of acetylation in the regulation of antimicrobial peptide gene expression in the human intestine"

Par Natalie Fischer

Thèse de doctorat de microbiologie

Dirigée par Philippe Sansonetti

Présentée et soutenue publiquement le 23 Septembre 2014

Devant un jury composé de :

ARLET, Guillaume	Professeur à l'UMPC (Président du jury)
CERF-BENSUSSAN, Nadine	DRCE INSERM (Rapporteur)
AIT-SI-ALI, Slimane	DR2 CNRS (Rapporteur)
SANSONETTI, Philippe	Professeur au Collège de France (Directeur de Thèse)
SPERANDIO, Brice	CR1 INSERM (Co-directeur de Thèse)

Acknowledgments

First of all, I want to thank Philippe Sansonetti for flying me in from San Francisco to interview and giving me the change to conduct a thesis in his laboratory. I enjoyed very much the scientific discussions, which we shared over my project and his input in my work. Also I want to thank him for the excellent resources concerning working conditions as well as education, which are provided in his lab. I am very thankful to have had the chance to work in the presence of his scientific genius and kindness and I am grateful for his support throughout my thesis and afterwards.

I want to thank Brice Sperandio for being my close supervisor for the past years. He guided me with the perfect balance of supervision, helped me settle in into the lab and project in the beginning, and later trusted me to walk on my own feet. I learned a lot from him, in the wet lab, but also concerning scientific writing and preparation of presentations. I am thankful that he respected my opinion and critics, encouraged my ideas and always had my best interest in mind. I am happy to say, that I think we made a good team and he really did a great job with me.

I especially want to thank everybody who agreed to be on my thesis jury, for their time and interest in my work. Special thanks go to Nadine Cerf-Bensussan and Slimane Ait-Si-Ali for taking the time to read and correct my work and to Guillaume Arlet, who kindly accepted to be the president of my jury. I am very much looking forward to discuss my work with you!

My time at the Sansonetti lab would not have been the same without the friendly atmosphere at PMM. I want to thank all my colleagues for their support, be it scientific discussions, technical help in the lab or mental with the beer after work. Especially I want to thank Emmanuel Sechet for his help in the last months, I could have never done all those Western Blots without him.

I would not have been here if it wasn’t for the EIMID ITN program, who gladly accepted me as a fellow. I enjoyed so much the international environment of this program. In this regard, I want to thank especially Marco Bargagna, who was always helpful and supportive, when it came to organizational things and deliverables. Also I want to thank the great group of EIMID fellows, who really made these years of my PhD special and with whom I enjoyed so many great times all around Europe.

I also want to thank my friends in Paris, especially the 12:30 lunch gang. Everybody knows that science is a tough street and that especially during a PhD some breaking points are reached. Thankfully I had a great support from my friends here at Pasteur and I will never forget the times we shared in the ville de lumières. A special thanks goes to Mathieu, who shared a very special year with me, and who has always was a great support and source for laughter and good times.

My family deserves a great round of applause for always supporting me during all my university education, morally and financially. I cannot thank you enough. I would not be here today if it wasn’t for my Mum, Meikel, my uncle Dieter and my dearly missed grandparents. This PhD title essentially goes to you!

List of Abbreviations

1,25D3	–	1,25-dihydroxyvitamin D_3
AD	–	Atopic dermatitis
AMP	–	Antimicrobial peptide
ANG4	–	Angiogenin 4
AP1	–	Activator protein 1
APC	–	Antigen-resenting cell
APRIL	–	A proliferation-inducing ligand
ARE	–	Apical recycling endosome
ATF2	–	Activating transcription factor 2
ATG16L1	–	Autophagy related 16 like 1
ATP	–	Adenosine triphosphate
BAFF	–	B-cell activating factor
BMK	–	Big mitogen-activated protein kinase
Brd4	–	Bromodomain-containing protein 4
CAMP	–	Cathelicidin antimicrobial peptide
CBP	–	cAMP-responsive element binding protein-binding protein
CCL20	–	CC-chemokine ligand 20
CCR6	–	CC-chemokine receptor 6
CD	–	Crohn’s disease
CDK9	–	Cyclin-dependent kinase 9
CDRE	–	Caudal responsive element
CFTR	–	Cystic fibrosis transmembrane conductance regulator
CHD	–	Chromodomain-helicase-DNA-binding protein
CNV	–	Copy number variation
CNS1	–	Conserved non-coding sequence 1
COPD	–	Chronic obstructive pulmonary disease
CRAMP	–	Cathelin-related antimicrobial peptide
CREB	–	Cyclic-AMP response element-binding protein
CRS	–	Cryptdins-related sequence
CTD	–	Carboxy-terminal domain
CTLA-4	–	Cytotoxic T-lymphocyte antigen 4
CXCR4	–	C-X-C chemokine receptor type 4
DC	–	Dendritic cell

DEFB1	–	Human beta defensin 2
DEFB2	–	Human beta defensin 2
DEFB3	–	Human beta defensin 3
DEFB4	–	Human beta defensin 4
DNA	–	Deoxyribonucleic acid
DNMT	–	DNA methyltransferases
E. coli	–	*Escherichia coli*
E. faecalis	–	*Enterococcus faecalis*
EGFR	–	Epithelial growth factor receptor
ER	–	Estrogen receptor
ERE	–	Estrogen-responsive element
ERK	–	Extracellular signal-regulated kinase
FAE	–	Follicle-associated epithelia
FliC	–	Flagella filament structural protein
FOXO	–	Forkhead box transcription factor
Foxp3	–	Forkhead box P3
FRPL1	–	Formyl peptide receptor-like 1
GALT	–	Gut-associated lymphoid tissue
GAS	–	Group A *Streptococcus*
GBS	–	Group B *Streptococcus*
GCN5	–	General control non-repressible synthesis 5
GNAT	–	GCN5-related *N*-acetyltransferases
HAT	–	Histone acetyltransferase
HBO1	–	HAT bound to Orc1
hCAP18	–	Human cationic antibacterial protein of 18 kDa
HD 5	–	Human alpha defensin 5
HD 6	–	Human alpha defensin 6
HDAC	–	Histone deacetylase
HDACi	–	HDAC inhibitor
HDM	–	Histone demethylase
HIF1	–	Hypoxia inducible factor 1
HIP/PAP	–	Hepatointestinal pancreatic/pancreatitis- associated protein
HIV	–	Human immunodeficiency virus
HMT	–	Histone methyltransferase

HNP	–	Human neutrophil peptide
HOTAIR	–	Homeobox antisense intergenic RNA
HPA axis	–	Hypothalamus–pituitary–adrenal axis
H. pylori	–	*Helicobacter pylori*
HSV	–	Herpes simplex virus
HRE	–	HIF1 responsive element
IAP	–	Intestinal alkaline phosphatase
IBD	–	Inflammatory bowel disease
IEC	–	Intestinal epithelial cell
IEL	–	Intraepithelial lymphocyte
IgA	–	Immunoglobulin A
IgG	–	Immunoglobulin G
IκBα	–	Inhibitor of NF-κB alpha
IKK	–	IκB kinase
IL8	–	Interleukin 8
IL10	–	Interleukin 10
IL1β	–	Interleukin 1 beta
ILF	–	Isolated lymphoid follicles
ILS	–	Insulin/insulin-like growth factor signaling
Imd pathway	–	Immune deficiency pathway
INF	–	Inflammatory gene
IRAK	–	Interleukin-1 receptor-associated kinase
IRF	–	Interferon response factor
ISWI	–	Imitation SWI
JDP	–	Jun dimerization protein
JNK	–	c-Jun N-terminal kinases
KCNN4	–	Calcium-activated potassium channel protein 4
KDM2A	–	Lysine-specific demethylase 2A
Lgr5	–	Leucine-rich repeat-containing G-protein coupled receptor 5
lincRNA	–	Large multi-exonic intervening non-coding RNA
LINoCR	–	LPS-inducible non-coding RNA
L. monocytogenes	–	*Listeria monocytogenes*
LPS	–	Lipopolysaccharide
LSD1	–	Lysine-specific demethylase 1
LTi cells	–	Lymphoid tissue inducer cells

mAchR	–	Muscarinic acetylcholine receptor
MAL	–	MYD88-adaptor-like protein
MAMP	–	Microbe-associated molecular pattern
MAPK	–	Mitogen-activated protein kinase
M cells	–	Microfold cells
MCP	–	Macrophage attractant protein
MDP	–	Muramyl dipeptide
MLN	–	Mesenteric lymph node
MOF	–	Males absent on the first
MORF	–	MOZ-related factor
MOZ	–	Monocytic leukemia zinc finger protein
MSK	–	Mitogen- and stress activated protein kinase
MSV	–	Multi site variations
M. tuberculosis	–	*Mycobacterium tuberculosis*
MUC-2	–	Mucin 2
MYD88	–	Myeloid differentiation primary response 88
nAchR	–	Nicotinic acetylcholine receptor
NCoR	–	Nuclear receptor corepressor
NEMO	–	NF-κB essential modulator
NFAT	–	Nuclear factor of activated T-cells
NF-κB	–	Nuclear factor kappa B
NK	–	Natural killer
NLR	–	NOD-like receptor
NOD	–	Nucleotide-binding oligomerization domain containing protein
NuRD	–	Nucleosome remodeling deacetylase
p300	–	E1A-associated protein of 300 kDa
P. aeruginosa	–	*Pseudomonas aeruginosa*
PCAF	–	p300/CBP-associated factor
PD1	–	Programmed cell death protein 1
pDC	–	Plasmocytoid DC
PDF	–	Plant defensin
PGE2	–	Prostaglandin 2
PGLYRP	–	Peptidoglycan recognition protein
PIC	–	Pre-initiation complex

PLA2s	–	Secreted phospholipase A2
Pol II	–	RNA polymerase II
PP	–	Peyer's patch
PPAR- γ	–	Peroxisome proliferator-activated receptor gamma
PRR	–	Pattern recognition receptor
PSA	–	Polysaccharide A
P-TEFb	–	Positive transcription elongation factor
REG3	–	Regenerating islet-derived protein 3
RHD	–	Rel homology domain
RIP2	–	Receptor-interacting protein 2
ROS	–	Reactive oxygen species
RNA	–	Ribonucleic acid
RTD	–	Rhesus teta defensin
SAGA	–	Spt-Ada-Gcn5 acetyltransferase
SAHA	–	Suberoylanilide hydroxamic acid
S. aureus	–	*Staphylococcus aureus*
SCFA	–	Short-chain fatty acid
SCTE	–	Stratum corneum tryptic enzyme
S. epidermidis	–	*Staphylococcus epidermidis*
SFB	–	Segmented filamentous bacteria
S. flexneri	–	*Shigella flexneri*
SIGIRR	–	Single immunoglobulin IL1R-related molecule
Sirtuins	–	Silent information regulator 2-related proteins
SLP1	–	SUN-like Protein 1
SLPI	–	Secretory leukocyte peptidase inhibior
SMRT	–	Silencing mediator of retinoic acid and thyroid hormone receptors
SNP	–	Single nucleotide polymorphism
SOCS	–	Suppressor of cytokine signaling
SP1	–	Specificity protein 1
S. pneumoniae	–	*Streptococcus pneumoniae*
STAT	–	Signal transducer and activator of transcription
S. typhimurium	–	*Salmonella typhimurium*
SWI/SNF	–	SWItch/sucrose non fermentable
TAD	–	Transactivation domain

TF	–	Transcription factor
TGFβ	–	Transforming growth factor beta
Th cell	–	T helper cell
Tip60	–	Tat-interacting protein of 60 kDa
TLR	–	Toll-like receptor
TNFα	–	Tumor necrosis factor alpha
TOLLIP	–	Toll interacting protein
TRAF	–	TNF receptor-associated factors
TRAM	–	TRIF-related adaptor molecule
Treg cell	–	Regulatory T cell
TRIF	–	TIR domain-containing adaptor protein inducing IFNβ
TSA	–	Trichostatin A
TSLP	–	Thymic stromal lymphopoietin
TSS	–	Transcriptional start site
UC	–	Ulcerative colitis
UHRF1	–	Ubiquitin-like, containing PHD and RING finger domains 1
VDR	–	Vitamin D receptor
VDRE	–	Vitamin D responsive element
Wnt	–	Wingless-type
XPB	–	Xeroderma pigmentosum B
XBP1	–	X-box binding protein 1
XIST	–	X-inactive specific transcript

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ANNEX 291

Introduction

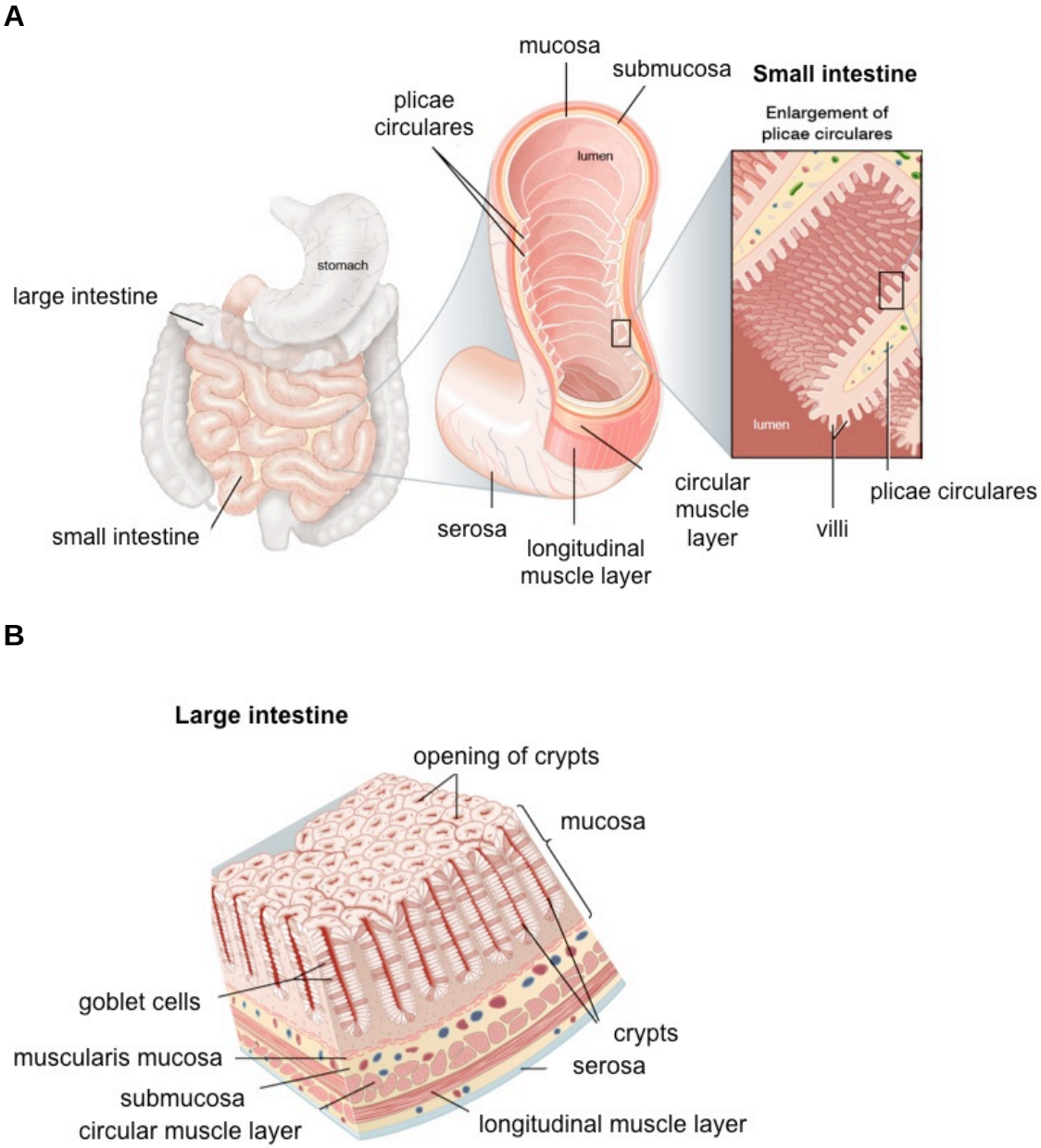


Figure 1: The human intestine. The human intestine is divided into the small intestine (A) and the large intestine (B). Several muscular layers, covered by the submucosa and mucosa, structure the wall of the intestine. The mucous membrane in the small intestine folds into the so-called plicae circulares. The surface of these folds is furthermore covered by villi to increase the total area for absorption. The mucosa of the large intestine contains crypts but does not possess villi. It is also lined with mucus-secreting goblet cells. (Adapted from Encyclopaedia Britannica, Inc. 2003)

Chapter I – Homeostasis in the human gut

I.1 The human gut

The 8 meter long intestine extends from the stomach to the anus and can be divided into the small intestine (subdivided into duodenum, jejunum and ileum) and the large intestine (subdivided into cecum, colon and rectum) [1] (Figure 1). The predominant task of the intestine is the processing of food coming from the stomach. The main part of digestion and absorption of nutrients takes place in the small intestine. Folding of the intestinal mucosa produces finger-like protrusions, the so-called villi, and invaginations, the so-called crypts, and allows an enlargement of the surface to a size of 250 m^2 for maximized absorption (Figure 1 A). In the much shorter, but wider large intestine we find crypts but no villi (Figure 1 B). Here water and salts are absorbed from the remnants of the small intestine and indigestible waste products are prepared for disposal. It is also the place with the highest density of bacteria, which help digest fiber and produce vitamins that are absorbed by the host. The wall of the intestinal tube is composed of four concentric tissue layers. The outmost layer of the intestinal tube is build of connective tissue, followed by a layer of several smooth muscle sheets, which allow the rhythmic contractions of the peristaltic to move the contents along the intestinal tract. Underneath the muscle layer we find the submucosa, consisting of connective tissue interspersed with nerve fibers and blood and lymphatic vessels, followed by the mucosa, which comprises the epithelium and the lamina propria. The lamina propria is a layer of connective tissue containing nerve fibers, blood and lymphatic vessels. An important feature of the lamina propria is its abundance of immune cells, such as lymphocytes and myeloid cells.

I.2 The intestinal epithelium

The epithelium of the intestine is in direct contact with the lumen and its contents. It consists of a single-cell layer of different specialized intestinal epithelial cells (IECs), which possess a vigorous rate of renewal (3-5 days) [2] (Figure 2 A). (i) Multipotent Leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5)-positive stem

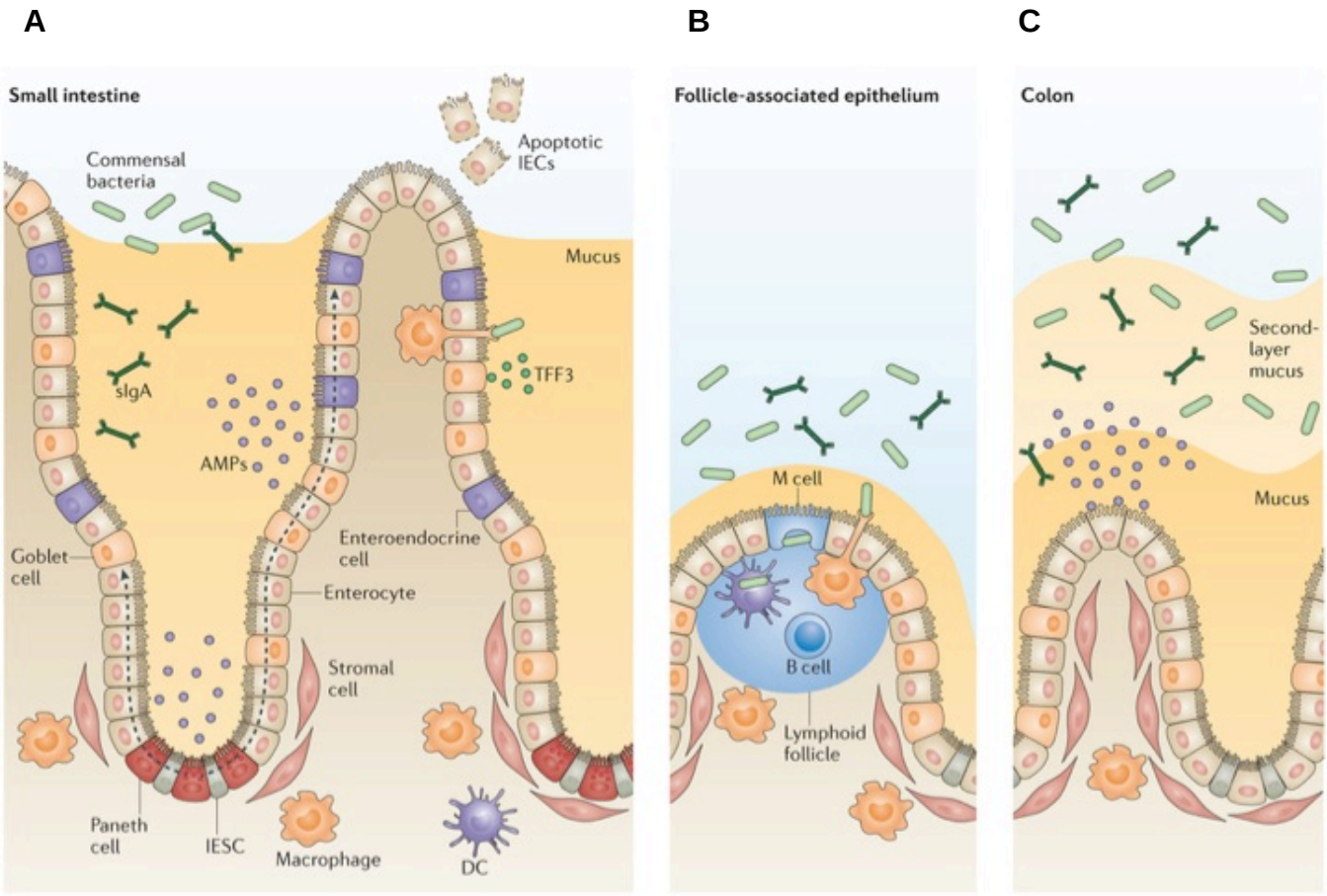


Figure 2: The intestinal epithelium. The intestinal epithelium is comprised of a variety of IECs originating from stem cells in the intestinal epithelial stem cell (IESC) niche in the crypt (A). Differentiated IECs then migrate upwards to renew the epithelium, as indicated by the dashed arrows. Microfold cells (M cells) in the follicle associated epithelium and goblet cells promote sampling of luminal antigens and live bacteria by resident dendritic cells (DCs) and intestine-resident macrophages, which reside in the underlying lamina propria (B). Furthermore, goblet cells secrete mucus covering the epithelium in a protective barrier (C). Transcytosed IgA antibodies reinforce this barrier together with antimicrobial peptides (AMPs) produced by enterocytes and/or Paneth cells, interspersed between the stem cells in the crypt [2].

cells are located in the crypts of Lieberkuhn and are capable to constitute all 5 IEC lineages, by constant proliferation and migration towards the villi tips in the small intestine or the surface of the epithelial cuff in the colon [3,4]. There, they replace terminally differentiated cells, which undergo apoptosis and exfoliation into the lumen. Furthermore, IECs are polarized, possessing two discrete membrane regions, the apical and basolateral side. The apical side faces the lumen, while the basolateral side is in contact with the underlying lamina propria. (ii) Enterocytes are the most abundant IECs in the small intestine and are responsible for the absorption of nutrients and the secretion of hydrolytic enzymes and immune mediators (i.e.: Antimicrobial peptides (AMPs)) into the lumen. (iii) Hormone producing enteroendocrine cells make up about only 1% of the cells in the epithelium [5]. They secrete various hormones, such as serotonin, vasoactive intestinal peptide and somatostatin, which regulate fluid and electrolyte secretion, motility, blood flow and food intake [6]. (iv) Paneth cells can only be found in the crypts of the small intestine, where they are interspersed with stem cells [7]. They are well recognized for their secretion of antimicrobial peptides, such as human alpha defensin (HD)5 and HD6, lysozyme, secretory phospholipase A2 (PLA2s) and c-type lectins. (v) Microfold (M) cells are preferably located in the so-called follicle-associated epithelia (FAE) right above the gut-associated lymphoid tissues (GALT), which is comprised of mesenteric lymph nodes (MLNs), isolated lymphoid follicles (ILFs) and Peyer's patches (PP) [8] (Figure 2 B). Their unique morphological features, such as reduced glycocalyx, an irregular brush border and broader microfolds instead of microvilli makes them highly specialized for phagocytosis and transcytosis of antigens from the gut lumen across the epithelial barrier [9]. On the basolateral side of the epithelium the sampled antigens are taken up by APCs and can be presented to T- and B-lymphocytes in the GALT. (vi) Goblet cells secrete heavily glycosylated gel-forming mucins that build up the layers of mucus covering the epithelium [10]. Mucin 2 (MUC2) is the main component of the mucus layer and forms a large, net-like polymer. The inner layer is firmly adherent to the epithelial cells and approximately 50µm thick. It possesses a dense structure, which does not allow bacteria to penetrate [11]. The second non-attached layer, which we find in the colon, is approximately 100µm thick (Figure 2 C). Its expanded volume allows few bacterial species to adhere and use the mucins as nutrient source [12]. The abundance of goblet cells, and with it the thickness of the

mucus layer increases towards the large intestine as the number of bacteria increases and the luminal content becomes more compact. Recent results suggest a role for goblet cells in the sampling of luminal antigens for antigen presenting cells (APCs) in the lamina propria [13].

I.3 The human microbiota

We share our world and our daily life with trillions of microscopic organism; among them are bacteria, viruses, archae, protozoa and fungi. They inhabit next to every terrain and water system of our planet [14]. But most importantly they colonize the surfaces of our own body, outnumbering our own somatic and germ cells by a factor of ten. They can be found on our skin and on all mucosal surfaces that interface with the environment, such as the gastrointestinal tract, the respiratory tract, the vagina, the nasal and oral mucosa and the eye [15]. The totality of organisms, which live in the ecosystem of their human host are called “microbiota”. This coexistence is usually commensal or mutually beneficial, but can also include pathogens, which can have a damaging influence on their host. The microbial diversity harbored by the human body has recently been explored in extensive international multicenter studies such as the NIH Human Microbiome Project [16]. Here 16S rRNA gene sequencing and metagenomic sequencing was performed on samples from 15-18 body sites on three separate visits of 242 healthy adults in the United States. The aim of this extraordinary project is to understand the microbial components of the human genetic and metabolic landscape and their contribution to healthy physiology and predisposition to disease. The ultimate goal is to define parameters for the manipulation of the human microbiota, which allows optimization of its performance in the context of an individual’s physiology. The following chapters will discuss the development and composition of the human gut microbiome and the interplay between microbiota and the host.

Among the mucosal tissues in the human body, the human gut harbors the most diverse and extensive ecosystem of microbiota. In the Human Microbiome Project the gut showed the greatest microbial diversity between individuals, but also the lowest variability in between visits [17] (Figure 3A). Nonetheless, the composition of the gut microbiome undergoes profound changes throughout the lifespan of an

individual (Figure 3B). Although the infant has been considered sterile, it seems like the first bacterial contact occurs already within the mother's womb in the amniotic fluid [18]. A recent paper described the presence of a unique microbiome within the placenta [19]. After birth the colonization depends highly on the mode of delivery and the associated contact with environmental microorganisms [20]. Babies delivered by cesarean section show lower bacterial counts and their microbiota resemble the microbiota of the mothers skin. On the other hand the microbiota of babies born naturally via the birth channel resemble the microbiota of the mothers vagina. Brest feeding presents an additional mode of colonization by microorganism present in the maternal milk. The first colonizers in the gastrointestinal tract of newborns are facultative anaerobic bacteria such as Proteobacteria. They are thought to adjust the highly oxidative environment for anaerobic species by decreasing the oxygen concentration. Thereby they allow successive colonization by anaerobic microorganisms such as Bacteroides, Actinobacteria and Firmicutes [21]. During the first year of life, the intestinal microbiota composition is simple and shows great variability between individuals and over time. The diversity increases exponentially throughout the first 3 years of life, where the microbiome finally reaches an "adult-like" state [20,22].

In 2011 Arumugam *et al.* suggested the clustering of adult humans into three distinct "enterotypes", depending on high presence of *Bacteroides*, *Prevotella*, or *Ruminococcus*, respectively [23]. This concept caused a lot of discussions in the field and the boundaries between the enterotypes may be fuzzier than earlier work suggested [24]. The composition of gut microbiota between individuals is highly related to the host's genetic disposition, diet, lifestyle, hygiene and the use of antibiotics [21,25]. 500-1000 different bacterial species have been identified in the intestine, which form a community of 10^{14} organisms. Firmicutes and Bacteriodetes are the two predominant intestinal phyla [26]. Members of the Firmicutes belong to Clostridia, Mollicutes and Bacilli, including Enterococci, Lactobacilli and Lactococci. Bacteroidetes are represented by Bacteroides species such as *B. thetaiotaomicron*, *B. fragilis*, *B. ovatus* and *B. caccae*. Furthermore, we can find Proteobacteria, Verrumicrobacteria, Actinobacteria, Fusobacteria, Spirochaetes and species closely related to Cyanobacteria [28]. The density and diversity of bacteria increases

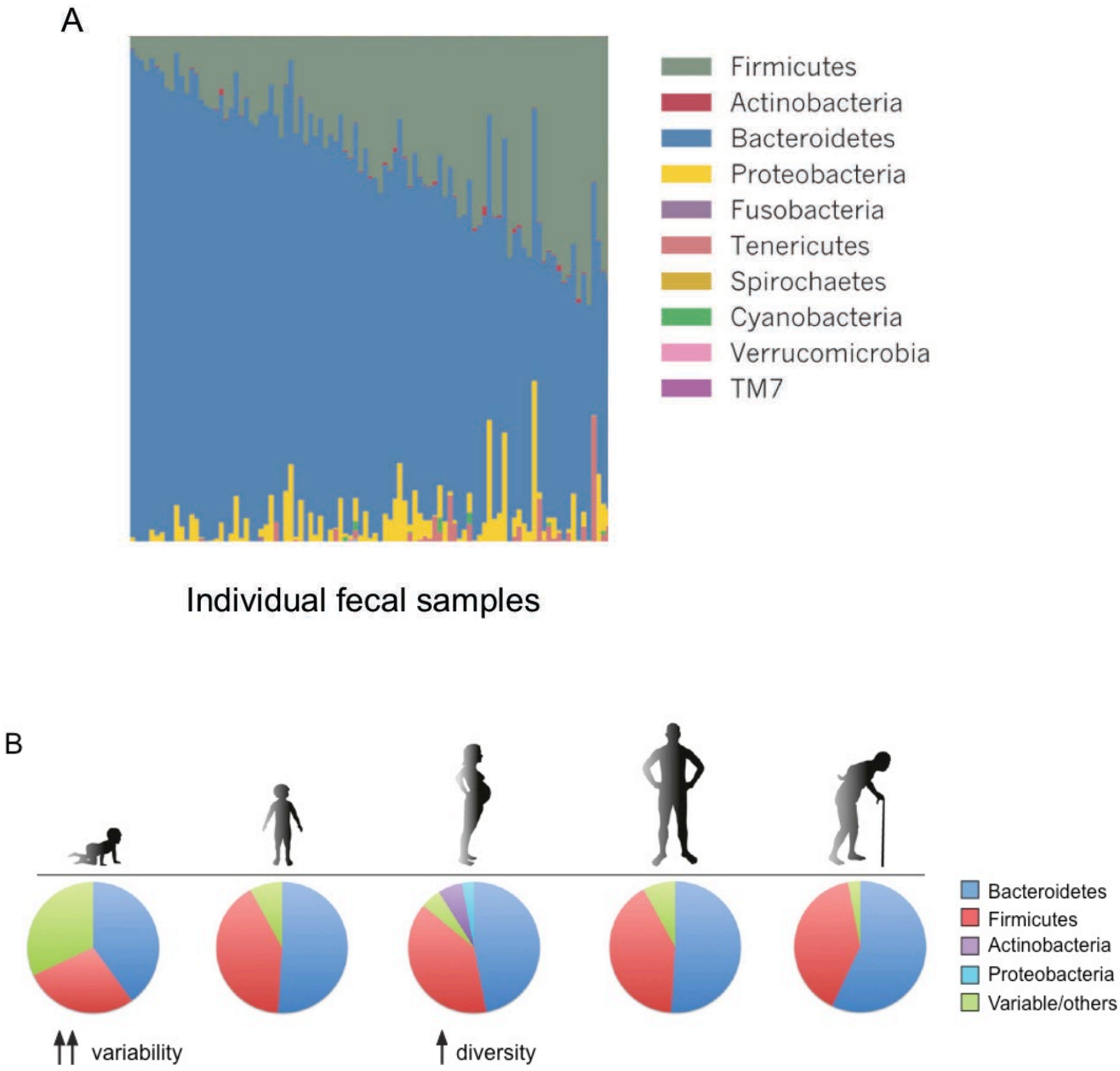


Figure 3: The composition of the human gut microbiota. Fecal communities analyzed using 16S rRNA show tremendous abundance diversity between individuals (A) (adapted from [17]). Interestingly, the variability, diversity and composition change during the lifespan of a human being, showing the highest variability within the first year (B) [27].

throughout the gastrointestinal tract from the stomach towards the colon. Most of them are located in the lumen of the gut. Nevertheless specific bacteria expressing lectins and glycosidases are able to adhere and inhabit the outer mucus layer and even use it as nutrient source [12]. Recent evidence suggests the existence of "crypt-specific core microbiota", as species of aerobic genera were found in the cecal and colonic crypts in mice [29].

I.4 How our gut microbiota shape our health

Over the recent years extensive efforts have been made towards unraveling the various connections between the gut microbiota and host physiology. Our microbiota provide us with a vast variability of gene products, which exceed the capabilities of the host alone. We refer to the collectivity of 5 million bacterial genes expressed in the intestine as the "microbiome". The huge metabolic capacity that goes along with those gene products has led to the view of the intestinal microbiota as an additional organ [30]. It is clear by now that intestinal microbiota are important supporters of our health and disturbances of the equilibrium of our coexistence contribute to a wide range of diseases, ranging from inflammation to obesity. This chapter will discuss the influences of gut microbiota on different aspects of host development and physiology.

I.4.1 Nutrition

The primary driving force behind the evolution of the mammalian gut microbiota appears to be alterations in host diets [31]. Colonization with microbiota provided a way to enhance the host's digestive efficiency [32]. The additional metabolic capacity of the microbiome is one of our biggest benefits from our invisible roommates. With help of bacterial enzymes we can metabolize otherwise indigestible polysaccharides from components of plant cell walls (including cellulose, xylan, and pectin) as well as undigested starch [33,35,36]. Bacteria convert released monosaccharides to pyruvate, which is then further processed by fermentation in the highly anaerobic environment of the distal intestinal lumen. The predominant end products of bacterial fermentation in the gut are short chain fatty acids (SCFA), such as acetate, propionate, and butyrate [37] (Figure 4). SCFA production represents 60–75% of the energy content of ingested carbohydrates. They are absorbed by passive diffusion across the

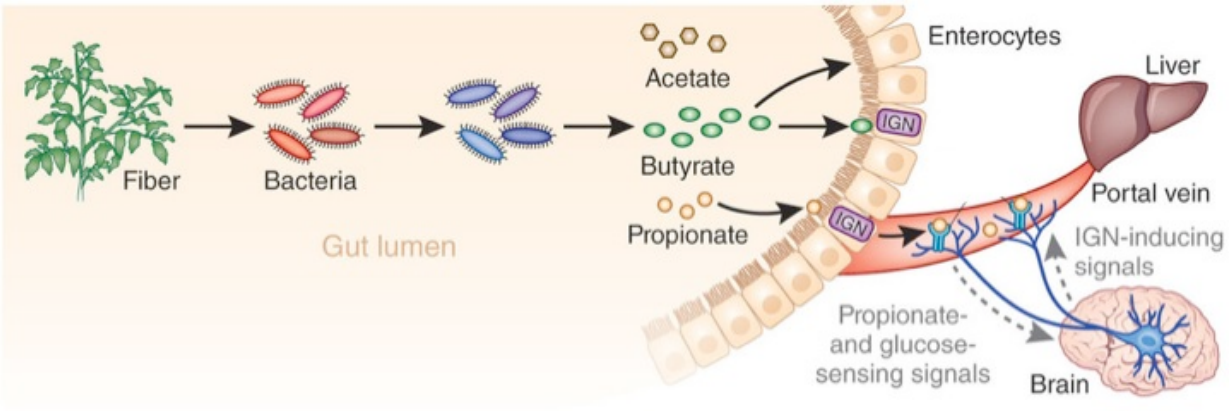


Figure 4: SCFA production by the gut microbiota. Indigestible fiber, such as plant cellulose, reaches the intestine where they are fermented by bacteria into the short-chain fatty acids acetate, propionate and butyrate. Butyrate is used by enterocytes as energy source, whereas acetate and propionate reach the liver by the portal vein. Enterocytes produce glucose, which is released into the portal vein. Propionate and butyrate additionally promote intestinal gluconeogenesis (IGN) via different mechanism. The resulting gut-to-brain communication conveys IGN-inducing signals back to the portal vein (adapted from [34]).

epithelium and diffused to different organs, or are used directly by the colonic epithelial cells. In addition to their nutritional value, SCFA have important effects on other aspects of gut physiology, for example uptake of water and salts, stimulation of intestinal blood flow, proliferation and differentiation of epithelial cells [38]. Interestingly, epidemiological data suggest that dietary fiber reduces the incidence of colorectal cancer, which could mean that microbiota produced SCFA help to maintain a healthy gut physiology [39,40]. Furthermore, our microbiota are involved in the production of certain amino acids (up to 20% of circulating plasma lysine and threonine in adults) and synthesis of vitamins, such as vitamin K, B_{12}, biotin, folic acid, and pantothenate [41,42].

Research on microbiota and obesity suggest a fundamental role for microbiota in the regulation of the host caloric energy balance and metabolic health [43] (Figure 5). A twin study could show that obesity is associated with changes in the microbiota on the phylum-level, reduced bacterial diversity and altered representation of bacterial genes and metabolic pathways [44]. Obese individuals show less species diversity and dominance of Firmicutes species, compared to non-obese individuals, which harbor a higher number of Bacteroidetes [45]. Moreover, a comparison between lean and obese mice suggests that the gut microbiota are able to influence the efficiency of energy harvest from the diet, as well as energy use and storage [21]. The microbiome of obese mice for example is enriched for genes encoding carbohydrate-processing enzymes and possesses a higher capacity for production of short-chain fatty acids. Germ-free mice on the other hand show reduced total body fat, even when fed a high fat and sugar “Western” diet [46]. Overall germ-free animals require a 30% higher caloric intake to achieve the same weight as conventional mice [47]. Taking the pathological scenario to another level, obesity often goes hand in hand with diabetes. Both those metabolic diseases are characterized by insulin resistance and a low-grade inflammation [48]. Cani and colleagues recently identified bacterial lipopolysaccharide (LPS) as a triggering factor for diabetes type 2 [49]. Mice fed with a high-fat diet showed chronic increase of the proportion of LPS- containing microbiota in the gut and LPS plasma concentrations. Furthermore, they could show that LPS infusion increased markers of inflammation and insulin resistance in the liver.

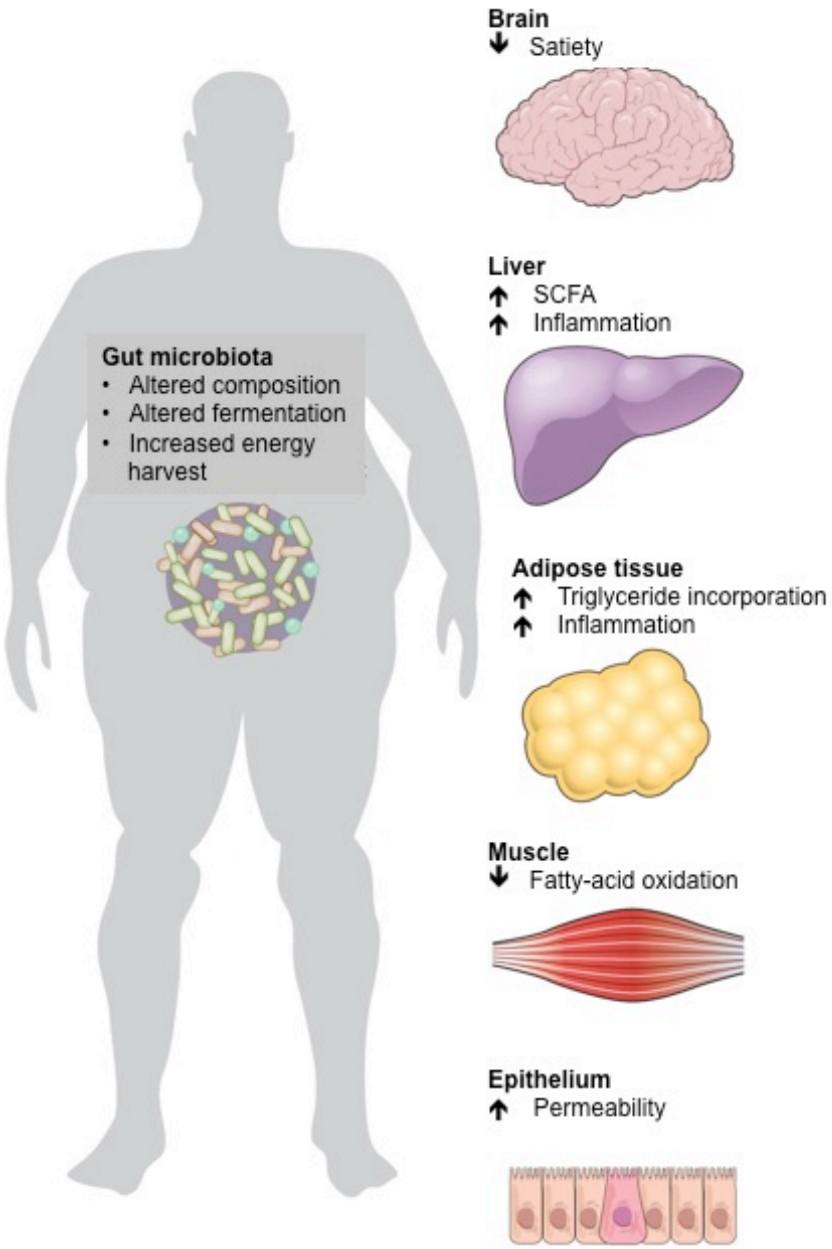


Figure 5: Microbiota in obese individuals. Alterations in the composition and metabolic capacity of gut microbiota in obese individuals promote adiposity and influence metabolic processes in peripheral organs, such as the control of satiety in the brain, the release of hormones from the gut, the synthesis and storage or metabolism of lipids in the adipose tissue, liver and muscle. Microbial molecules such as LPS also increase intestinal permeability, leading to systemic inflammation and insulin resistance (adapted from [32]).

I.4.2 Development and morphogenesis of host organs and structures

The gut microbiota have a significant influence on development and morphogenesis of host organs and physiological structures. The importance of microbiota in the maturation and shaping of the gastrointestinal tract can best be illustrated on the example of germ-free mice, which display considerable deficits [21]. Besides altered mucus thickness and properties, they have a reduced intestinal surface area as compared to conventional mice [50], due to reduced villi thickness and impaired brush border differentiation, resulting from slowed cell proliferation and turnover of epithelial cells [51-54] (Figure 6). Furthermore, decreased intestinal peristaltic activity leads to a prolonged transit time of food through the gastrointestinal tract [55,56]. Functional microbiota on the other hand, reduce epithelial permeability, thereby strengthening the barrier function, and positively influence the modeling of the vascular system [52].

Additionally, to influencing gut development microbiota have also been found to alter bone homeostasis [57]. Germ-free mice have a higher bone mineral density, reduced number of osteoclasts and lower levels of serotonin, a hormone that inhibits bone formation [58,59]. Microbial colonization promotes bone resorption via the activation of osteoclasts. Furthermore, it leads to an increase in the number of pro-inflammatory Th17 cells, which in turn induce osteoclastogenesis [60,61]. The suggested role for microbiota in the development of osteoporosis needs further research efforts.

The connection between microbiota and cardiac development needs further investigation, as it has only been described in one study so far. Germ-free mice were found to have a smaller heart in proportion to their body weight, in comparison to conventional mice [62].

I.4.3 Development and activity of the gut immune system

Besides, influencing the morphological development of the gut, the microbiota also shape the maturation of the local immune system. Interestingly, even single species have been described in their characteristic shaping of particular immunological settings.

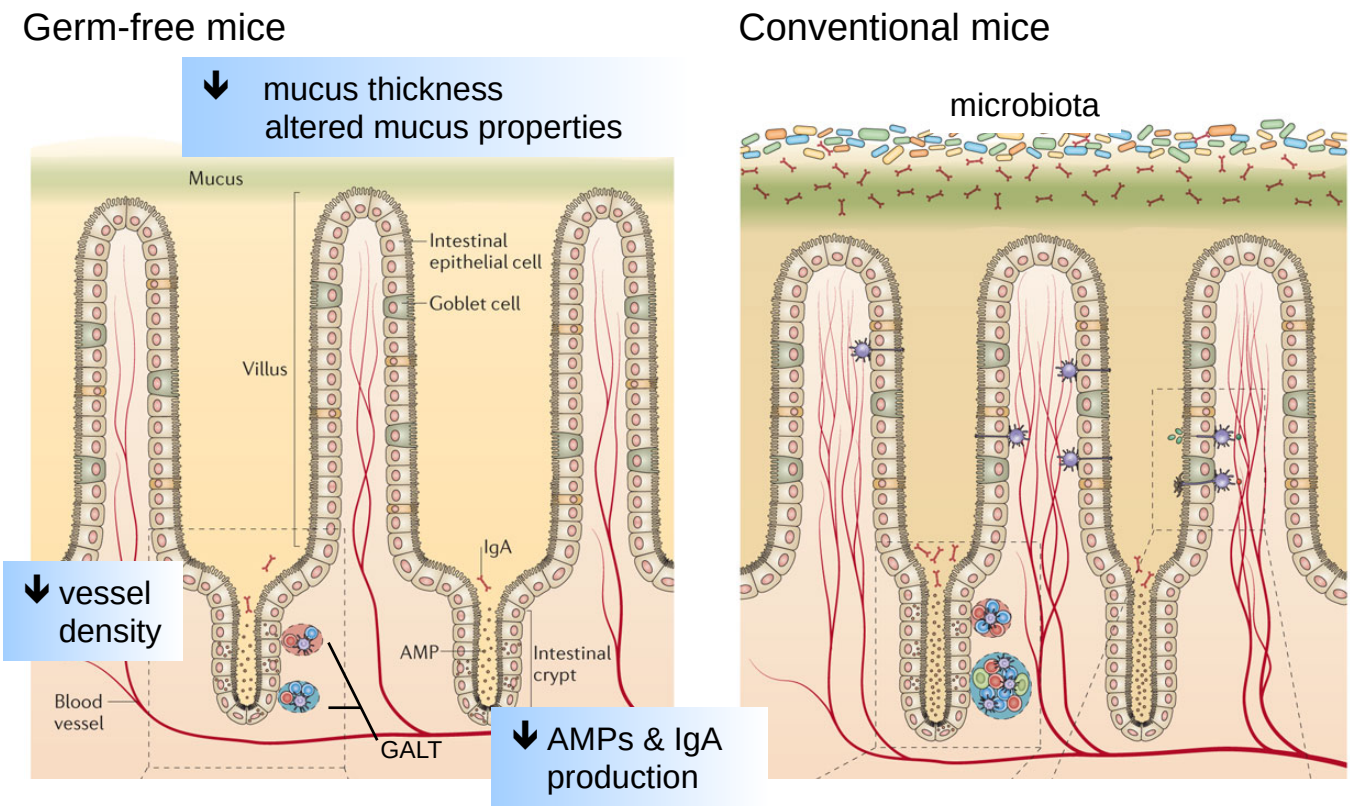


Figure 6: Deficits in intestinal morphological and immunological development in germ-free mice. The influence of microbiota on the gut morphological and immunological development can best be illustrated in the comparison of germ-free mice with conventional raised mice. Germ-free mice show longer and thinner villi in the distal small intestine, which have a less complex vascular network. Furthermore, their intestinal crypts are less deep and contain fewer proliferating stem cells. They possess reduced mucus thickness and altered mucus properties, along with reduced secretion of AMPs and IgA. Overall germ-free mice show severe immunological underdevelopment, such as few isolated lymphoid follicles, immature Peyer's patches and immature mesenteric lymph nodes (adapted from [21]).

1.4.3.1 Development of the GALT

Studies in germ-free mice showed that microbial colonization is essential for the maturation of gut-associated lymphoid tissues, as these structures are underdeveloped in germ-free mice [63] (Figure 6). A recent study could even show the maturation of ILFs is driven by a specific bacterial factor, the peptidoglycan of the bacterial cell wall. Bouskra *et al.* showed that the recognition of peptidoglycan by the host receptor nucleotide-binding oligomerization domain containing 1 (NOD1) in epithelial cells leads to expression of CC-chemokine ligand 20 (CCL20) and beta-defensin 3 (DEFB3). These molecules are in turn able to bind to CC-chemokine receptor 6 (CCR6) on lymphoid tissue inducer (LTi) cells, which then induce ILF formation [64].

1.4.3.2 Development of lymphoid cells

Furthermore, the microbiota direct the development of the lymphoid cell repertoire, via the induction of chemokine and cytokines produced by intestinal epithelial cells. They are important for example for the differentiation of natural killer (NK) like RORγt$^+$NKp46$^+$ cells, which have been found to be scarce in germ-free mice [65]. These cells produce interleukin 22, which strengthens the intestinal barrier integrity, by promotion of epithelial repair and production of AMPs [66]. On the contrary, microbiota are able to limit the generation of a subset of pro-inflammatory NK cells, the so-called invariant NK T cells. Numbers of these cells are increased in germ-free mice [67]. Forkhead box P3 (Foxp3) positive regulatory CD4$^+$ T (Treg) cells possess anti-inflammatory properties and are important regulators of the intestinal homeostasis [68]. Conflicting observations have been reported for the influence of microbiota colonization on the development of those cells. Multiple studies reported a reduction of the Treg marker Foxp3 among CD4$^+$ T cell subsets in germ-free mice [71-73]. Moreover, Treg cells from germ-free mice produce less interleukin-10 (IL10) and are less sufficient in suppression of effector T cell proliferation. At the same time two contradicting studies reported no change in the percentage [74] or even an increase of CD4$^+$ FOXP3$^+$ cells in germ-free mice [75].

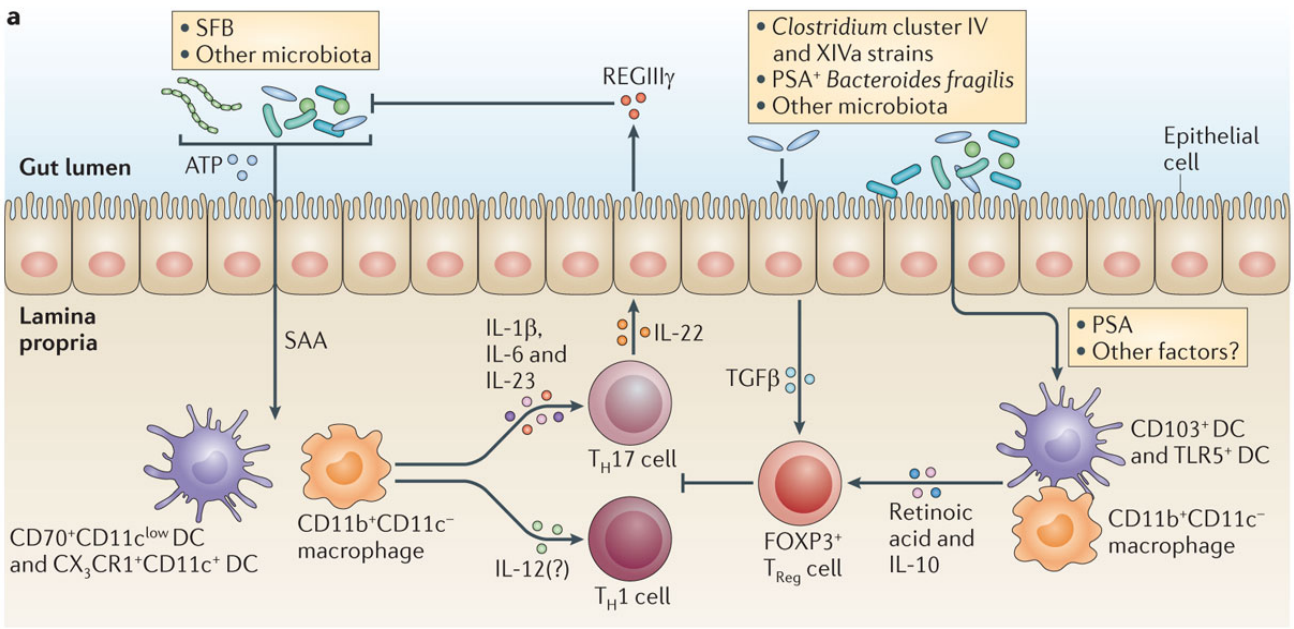


Figure 7: Shaping of distinct immune responses by certain microbiota. Certain bacterial species have been identified, which are able to shape the intestinal immune environment of their host. Segmented filamentous bacteria (SFB) influence lamina propria dendritic cells (DCs) and macrophages to induce pro-inflammatory T helper 17 (Th17) cells and Th1 cells. Furthermore, bacteria-derived adenosine-triphosphate (ATP) was shown to promote the development of Th17 cells via the activation of a subset of DCs, which produce IL1β, IL6 and IL23 [69].On the other hand Clostridium species and *Bacteroides fragilis* stimulate intestinal epithelial cells, T cells, and lamina propria DCs and macrophages to promote the development and/or the activation of anti-inflammatory regulatory T (Treg) cells (from [70]).

1.4.3.3 How distinct microbiota species shape differential immune responses

Interestingly, individual species of the gut microbiota have been found to shape either pro- or anti-inflammatory T cell related responses in the intestine (Figure 7). The differentiation and expansion of anti-inflammatory Treg cells was shown to be induced by *Bacteroides fragilis* polysaccharide A (PSA) via binding to Toll-like receptor (TLR) 2 on undifferentiated $CD4^+$ T cells [76]. Furthermore, a study in germ-free mice demonstrated expansion of Treg cells by colonization with 46 species belonging to *Clostridia*, which had been isolated from conventional mouse faeces [77]. On the contrary, a pro-inflammatory influence can be seen in mice colonized with segmented filamentous bacteria (SFB). These bacteria naturally colonizes the rodent intestine at the time of weaning and induce a prominent Th17 and Th1 response in their host [78,79]. Thereby they stimulate the postnatal maturation of the gut immune system and strengthen the epithelial barrier [80,81]. The induced Th1 and Th17 cells promote barrier function via recruitment and activation of macrophages and neutrophils and the stimulation of AMPs production by epithelial cells. Conventional mice lacking SFB show the absence of Th17 cells [78,79], elicit a lower IgA antibody response [82] and overall weaker intestinal T cell response [80]. The diminished immune capabilities make these mice more susceptible to infection with the rodent pathogen *Citrobacter rodentium* [79]. The signals send by SFB by which they induce Th17 and Th1 differentiation are not yet fully understood. Ivanov and colleagues could show that SFB induces the acute phase protein serum amyloid A protein (SAA), which in turn stimulates lamina propria DCs for Th17 generation [79]. Recent studies point towards a specific SFB antigen-directed mechanism [83,84]. SFB are in direct contact with the epithelium and even partially invade intestinal epithelial cells. Therefore the close attachment to the epithelium facilitates sampling and presentation of SFB antigens in Peyer's patches. Moreover, cell-contact mediated signaling cannot be excluded as a possibility. Whereas the close relation between SFB and an immunocompetent host is generally beneficial and protective, development of intestinal inflammation was shown in immunocompromised mice [85].

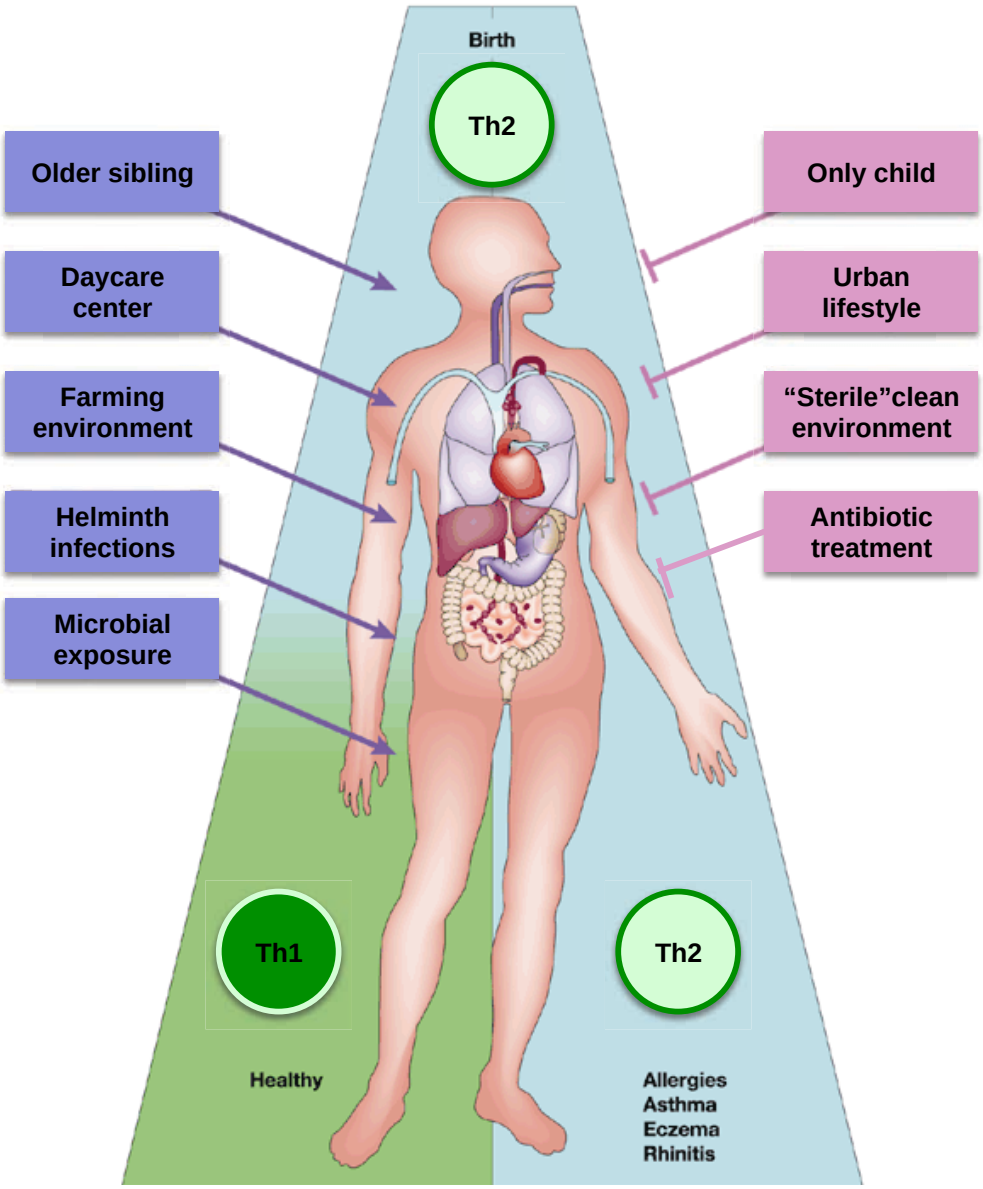


Figure 8: Microbial contact influences the development of appropriate systemic immune responses. The hygiene hypothesis states that the immature immune system at birth is skewed towards a Th2 response. Contact with microbes promotes a healthy development of the systemic immune response towards a balance of Th1 and Th2 responses. If this contact is lacking due to increased hygiene in a modern western civilization the immature Th2 state persists, leading to an increased risk of asthma and other atopic diseases (adapted from [100]).

1.4.3.4 Development of an adaptive B cell IgA response

Moreover, intestinal bacteria are important for the development of adaptive immune responses, such as the development and maturation of antibody producing B cells. Sensing of bacterial flagellin via TLR5 receptor on dendritic cells (DCs) in the lamina propria promotes the differentiation of B cells into IgA producing plasma cells [86]. IgA has a key role in barrier homeostasis as deficient mice show production of microbiota-specific IgG antibodies, a sign for bacterial breach of the epithelium [87]. On the other hand, IgA is an important player in shaping the composition of the gut microbiota [88]. Mice deficient in the programmed cell death protein 1 (PD1) possess an altered IgA repertoire, resulting in a shift in microbiota from *Bacteroides* and *Bifidobacterium* to Enterobacteriaceae.

1.4.3.5 Induction of mucus production

The constitution of the protective mucus layer is also supported by the presence of intestinal microbiota. Germ-free rats have fewer goblet cells and therefore a thinner mucus layer compared to conventional animals [89]. A conventional mucus layer can be developed in germ-free mice upon stimulation with bacterial products, such as LPS or peptidoglycan. Moreover, bacteria-produced SCFAs directly activate the expression MUC2, the main component of the mucus layer [90]. MUC2 deficient mice show bacterial overgrowth and develop spontaneous colitis [91].

I.4.4 Development and activity of systemic immunity

In 1989 David Strachan formulated his much discussed “hygiene hypothesis”, declaring that reduced exposure to microorganisms during childhood in developed countries was the cause of increased incidence of hay fever, asthma and childhood eczema later in life [92]. This hypothesis proclaimed the idea that families with fewer children provided less possibility for cross infections and therefore insufficient microbial exposure. Additionally, the "hygiene revolution" of the 19th and 20th centuries brought important public health measures such as the collection of garbage, portable water, sanitation, vaccination and antibacterial therapeutics, which reduced the contact with infectious but also non-infectious microbes. In accordance with the hygiene hypothesis a lack of contact with microorganisms during development leads to skewing of the immune response away from a Th1 and towards a Th2 response and this imbalance leads to improper reactions related to

ectopic diseases and allergies [93] (Figure 8). Later Graham Rock proclaimed the new adapted “old friends hypothesis” [94]. He discussed the coevolution of commensal bacteria and their hosts, which were able to tolerate latent infections or carrier states. He especially emphasizes the need for microbes in the development of our immune system [95].

Studies comparing conventional and germ-free mice were able to highlight the importance of microbial colonization for the maturation of the systemic immune system. Notable deficits have been described in germ-free mice, such as reduced germinal center size in the spleen, decreased numbers of memory $CD4^+$ T cells, reduced numbers of Th1 cells in both systemic and mucosal compartments and a skewing towards a Th2-type cytokine profile [96-98]. It has been postulated that the maturating effect of microbiota beyond the gut are executed by soluble bacteria factors that cross the epithelial barrier and enter the bloodstream. Mazmanian and colleagues showed that monocolonization of germ-free animals with *B. fragilis* is sufficient to mediate establishment of a proper Th1/Th2 balance and lymphoid organogenesis and thereby corrects several immunologic defects found in the absence of a bacterial microflora [97]. These effects are established by PSA presented by intestinal DCs to activate CD4+ T cells, and elicit appropriate cytokine production. In another study Clarke and colleagues could show that peptidoglycan translocated from the gut systemically primes the innate immune system by activation of neutrophils in the bone marrow, enhancing their killing activity against *Streptococcus pneumoniae* and *Staphylococcus aureus* [99]. Serological peptidoglycan concentrations correlated directly with neutrophil function. Moreover, they could show that this effect was dependent on NOD1 signaling.

I.4.5 Protection against pathogens

The gut provides niches for all kinds of bacterial species, among other microorganisms. Nonetheless, there is a competition for space and nutrient supply. Occupation of these niches with commensal bacteria protects our intestine from more susceptible to infection with enteric pathogen, such as *Shigella flexneri* [101], *Listeria monocytogenes* [102] and *Salmonella typhimurium* [103]. Still pathogens have developed tricks to succeed in competition with the local microbiota. In order to successfully colonize the host *Salmonella* induces inflammation, which reshapes the microbiota

composition, allowing the pathogen to establish an infection [104]. A recent study showed that this induction of inflammation also allows *S. typhimurium* to overcome nutritional competition with the microbiota. Upon inflammation host cells release ethanolamine, which can be used as an energy source by *Salmonella* but not other competing species in the gut [105]. Not to forget the administration of antibiotics does also cause a disruption of the microbial balance in the gut and thereby leaves dangerous room for pathogen colonization [106].

I.4.6 Brain and behavior

The connection between the gut and the brain might not seem obvious; nevertheless multiple studies in germ-free and conventional mice have shown the influences of microbiota on brain function and behavior. There is accumulating evidence that microbiota influence the regulation of anxiety, mood, cognition and pain in their hosts [107,108]. The gut–brain axis presents a bidirectional communication system that integrates neural (vagus and enteric nervous system), endocrine (cortisol), and immunological (cytokines) signaling. In addition, the previously described SCFAs are neuroactive and can also modulate brain function and behavior [109] (Figure 9). Independent studies in different lineages of germ-free mice showed alterations in concentrations of neurotransmitters and neurotrophic factors in the brain, resulting in an altered stress response and less anxiety in elevated plus maze or light–dark box tests [110-112]. On the other hand, microbiota dysbiosis induced by infection with *Citrobacter rhodentium* or antibiotic treatment in conventional mice can cause anxiety-like behaviors [113]. The first study to show alterations at the cognitive level was undertaken by Gareau and colleagues. They showed deficits in simple non-spatial and working memory tasks, such as recognition of a novel object or spontaneous alterations in a T-maze, in germ-free mice [114]. Germ-free animals possess greater locomotor activity, linked with their resistance to obesity induction [46]. Other studies found a higher level of proteins involved in synaptogenesis in germ-free mice, suggesting an involvement of microbiota in synaptic connectivity [111,115].

Looking at the communication in the opposite direction, from the brain to the gut, stress and anxiety activate the hypothalamus–pituitary–adrenal (HPA) axis, which regulates cortisol secretion. Cortisol can affect cytokine secretion by immune cells, locally in the gut and systemically, as well as gut permeability and barrier

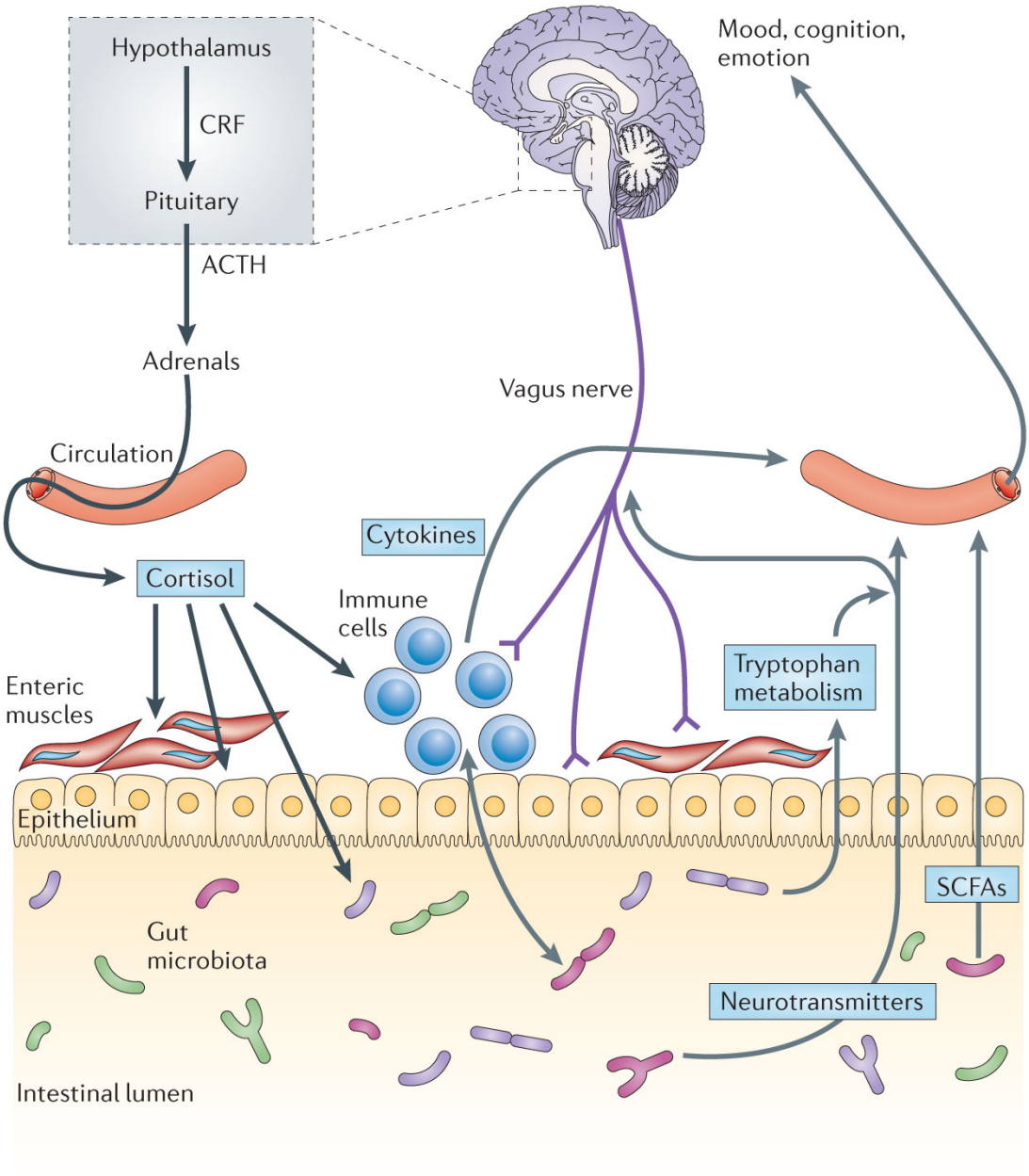


Figure 9: The gut-brain axis communication. The bidirectional communication between gut and brain is managed via endocrine (cortisol), immune (cytokines) and neural (vagus and enteric nervous system) pathways. In direction gut-to-brain the vagus nerve, modulation of systemic tryptophan levels and production of neuroactive SCFAs are strongly implicated in relaying the influence of the gut microbiota to modulate brain and behavior. The other way around the brain can influence the composition of the gut microbiota, induce cytokine secretion and alter gut permeability and barrier function, for example in stress situations via the release of cortisol ([107]).

function. Thereby stress and anxiety can cause changes in the intestinal microbiota, which in turn lead to gastrointestinal disorders, including irritable bowel syndrome (IBS) and inflammatory bowel disorder [116].

I.5 How we maintain intestinal homeostasis with our microbiota

The maintenance of intestinal homeostasis is a complex interplay between the local microbiota, the intestinal epithelium and the host immune system in the lamina propria. The trick is to achieve a balance between tolerance and unresponsiveness towards commensals and the readiness to launch an active immune response in the case of bacterial breach of the epithelial barrier. A state of tolerance is maintained by two mechanisms – ignorance towards commensal bacteria and constrain of effector cells in the case of contact with antigens. At the same time the microbiota commensal lifestyle supports this state of tolerance, as the majority of bacteria are located in the lumen of the intestine at a respectable distance from the host immune detection. Additionally, bacteria have developed special traits that help them to stay undetected or even allow active suppression of an immune response. This part will discuss the various mechanisms employed by the host, but also by the microbiota to support a life together in peace and symbiosis.

I.5.1 The mucus layer – an important first physical barrier

The most important and at the same time simplest mechanism to maintain tolerance is to avoid contact. The mucus layer covering the intestinal epithelium presents a potent physical barrier between the microbiota and the host tissue (Figure 10). Its physical properties have already been discussed in chapter I.2. The secretion of antimicrobial peptides by IECs and Paneth cells additionally increases the protective capacity of this barrier. These molecules accumulate in the mucus and build up a protective gradient towards the intestinal lumen [11,118]. This allows for the control of the numbers of mucosa-associated bacteria, but doesn't influence the overall luminal bacterial load. Furthermore, the mucus layer contains IgA antibodies, produced by B cells in the lamina propria and transcytosed across the epithelium [119]. IgA recognizes and traps bacteria in the mucus layer, which allows their subsequent removal by the peristaltic movement. It can also recruit factors of the complement system, which

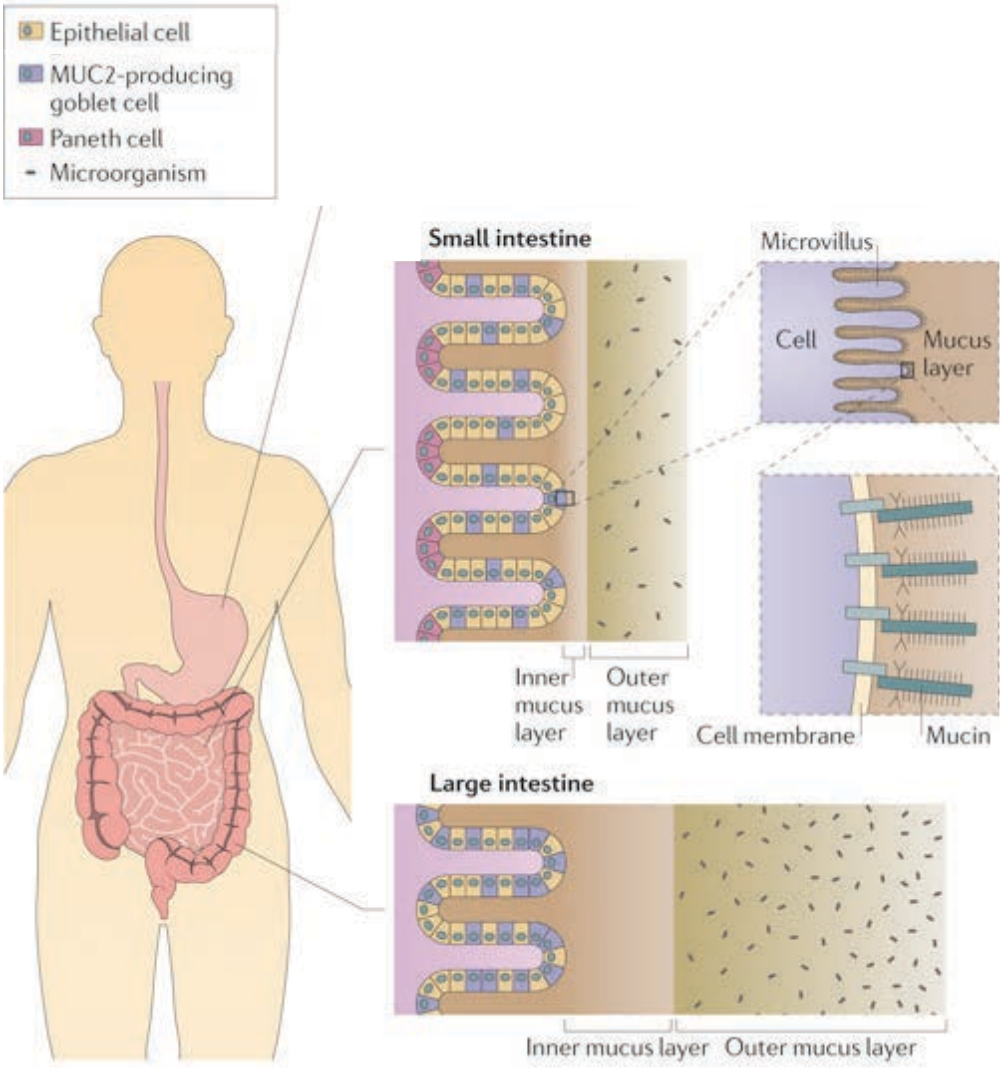


Figure 10: The intestinal mucus layer. In the small intestine mucus is produced by goblet cells and consists of an inner layer of ~15–30 µm and an outer layer of 100–400 µm. Additionally to the gel forming mucus layer, epithelial cells are covered with microvilli containing a high density of transmembrane cell surface mucins. In the large intestine mucus is predominantly produced by goblet cells and consists of a sterile inner layer of ~100 µm and a thick outer layer of ~700 µm (adapted from [117]).

promote bacterial lysis or mark bacteria for rapid phagocytosis and killing by lamina propria macrophages in the case of tissue invasion [120].

I.5.2 The intestinal epithelium – physical barrier, sensor and communicator

As the intestinal epithelium interfaces directly with the intestinal lumen and its microbial contents its role in the maintenance of homeostasis is uncompared. IECs employ a wide range of mechanism, which guarantee unresponsiveness and tolerance towards the microbiota. At the same time they are at guard and ready to alert the immune system in the case of an invasion. The following chapters will discuss the role of the intestinal epithelium as a barrier towards intestinal contents, sensor of microbial signals as well as pathogens and communicator towards the underlying immune system in the lamina propria.

I.5.2.1 The intestinal epithelial layer as physical barrier

The intestinal epithelial cell layer is composed of enterocytes, which are tightly linked to their neighboring cells via gap junctions. This blocks trespassing of any microbe across the epithelium. Moreover, the tight connection allows for gap-junctional intercellular communication and the horizontal forwarding of immune-receptor-mediated stimulations [121,122]. This might facilitate a coordinated antimicrobial response from the epithelium, but also offers the possibility of launching an immune response based on single “sensor cells”.

I.5.2.2 The intestinal epithelium as sensor of microbial signals

The intestinal epithelium is the main monitor and regulator of host-microbial interactions in the gut. Despite the physical protective capacity against bacterial translocation, microbial products can still diffuse through the mucus layer and can be sensed by innate immune receptors of the epithelium [124]. To avoid a constant inflammatory response a certain unresponsiveness of the epithelium in the case of contact with luminal microbial associated molecular patterns (MAMPs) has to be guaranteed. The following part aims to discuss the various ways to achieve epithelial unresponsiveness. The toll-like receptors (TLRs) are one of the best-studied families of innate immune pattern recognition receptors (PRRs). They are transmembrane receptors, localized either in the cell membrane or the membrane of intracellular endosomes. In humans 10 different TLRs have been identified, whereas mice

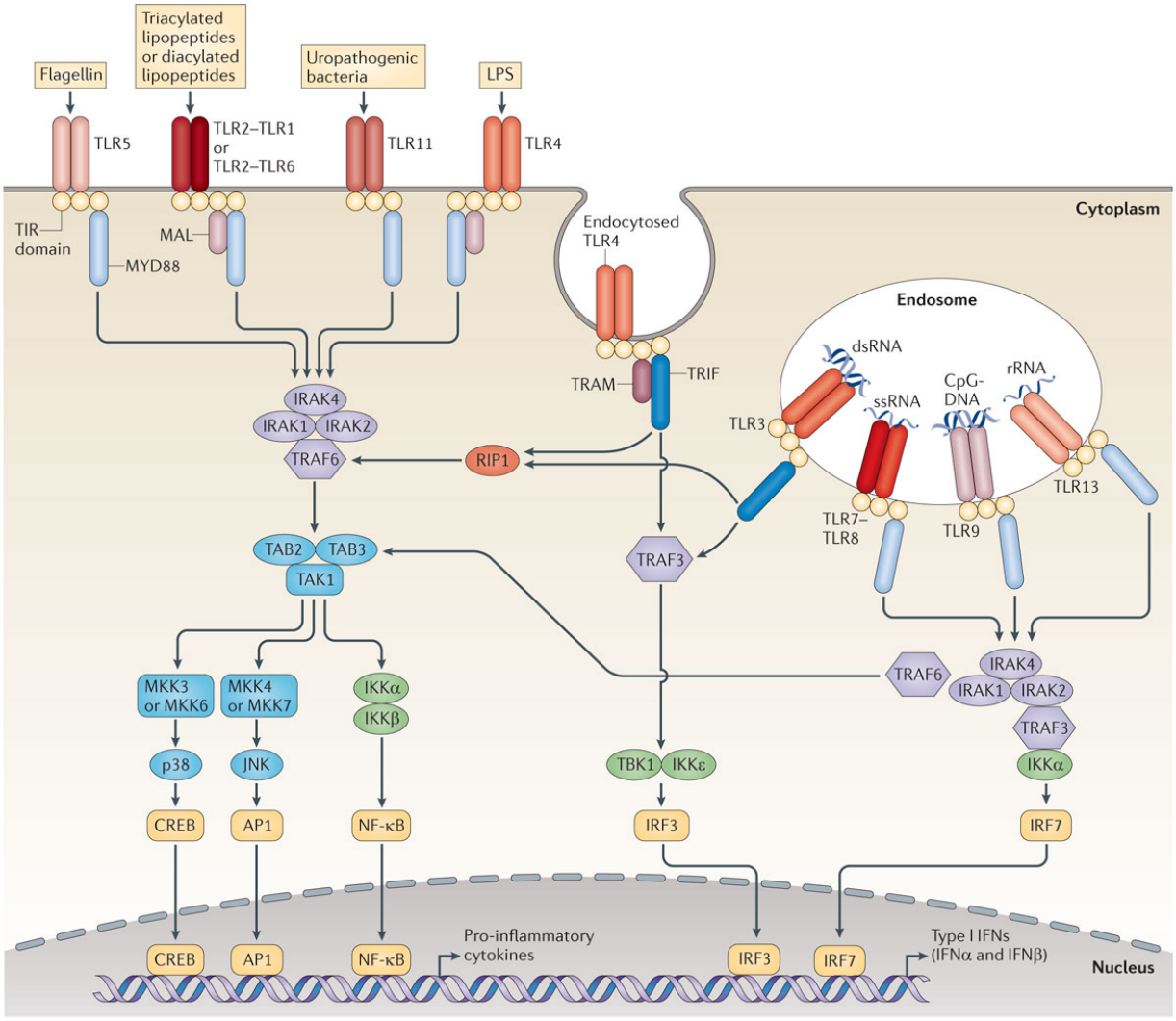


Figure 11: Toll-like receptor signaling. TLR5, TLR11, TLR4, and TLR2–TLR1 or TLR2–TLR6 are localized at the cell surface, whereas TLR3, TLR7–TLR8, TLR9 and TLR13 localize in endosomes. TLR4 localizes at both the plasma membrane and the endosomes. TLRs transmit their signals via various adapter molecules, such as MYD88, MYD88-adaptor-like protein (MAL), or TIR domain-containing adaptor protein inducing IFNβ (TRIF) and TRIF-related adaptor molecule (TRAM). This is followed by IL1R-associated kinases (IRAKs) and TNF receptor-associated factors (TRAFs) activation. Finally, mitogen-activated protein kinases (MAPKs) p38 and Jun N-terminal kinase (JNK) activate transcription factors cyclic AMP-responsive element-binding protein (CREB) and activator protein 1 (AP1). Together with the activation of transcription factors nuclear factor-κB (NF-κB) and the interferon-regulatory factors (IRFs), this leads to the expression of pro-inflammatory cytokines (from [123]).

Additionally, possess TLRs 11, 12 and 13. Each TLR is specialized for the recognition of certain microbial molecules [123,125] (Figure 11). TLR signaling is initiated by ligand-induced dimerization of receptors. Thereby they can signal either as homodimers, or as heterodimers, such as for example TLR2 with either TLR1 or TLR6. Following activation, the Toll–IL1-resistence (TIR) domains of TLRs engage TIR domain-containing adaptor proteins such as myeloid differentiation primary response 88 (MYD88), MYD88-adaptor-like protein (MAL), or TIR domain-containing adaptor protein inducing IFNβ (TRIF) and TRIF-related adaptor molecule (TRAM). The signal is then transmitted via IL1R-associated kinases (IRAKs) and the adaptor molecules TNF receptor-associated factors (TRAFs) and finally results in the activation of the mitogen-activated protein kinases (MAPKs) and transcription factors, which induce the expression of AMPs and pro-inflammatory cytokines and regulates various important cellular functions. Signaling via these receptors is crucial for the maintenance of intestinal barrier integrity, as it is involved in epithelial cell proliferation, IgA and AMP production and maintenance of gap junctions [126-128].

Countless studies in cell lines, mice and in human tissue have aimed to determine expression and regional and spatial localization of TLRs in the intestinal epithelium, producing somewhat different and sometimes contradicting results [129]. The level of expression of TLRs in the intestinal epithelium is generally low and their localization and compartmentalization differs from immune cells. Expression level and localization differs not only between the small and the large intestine, but also between epithelial cell types (Figure 12). Special compartmentalization is one of the main features to prevent an unjustifiable immune response. An example for this is the strictly basolateral localization of TLR5,which allows the detection of bacterial flagellin only in the case of a barrier breach [130]. This feature promotes both, unresponsiveness towards commensals on the apical side and at the same time detection of invading pathogens on the basolateral side. On the other hand TLR9, which is the receptor for unmethylated CpG sequences in bacterial DNA, is expressed on the apical and the basolateral side [131]. But depending on the side of the epithelium, activation elicits very distinct responses. Lee et al. showed that ligand binding on the apical side promotes tolerance, especially by inhibition of a response coordinated bythe transcription factor nucear factor kappa B (NF-kB). In contrast ligand binding on the basolateral side stimulates a strong proinflammatory cytokine

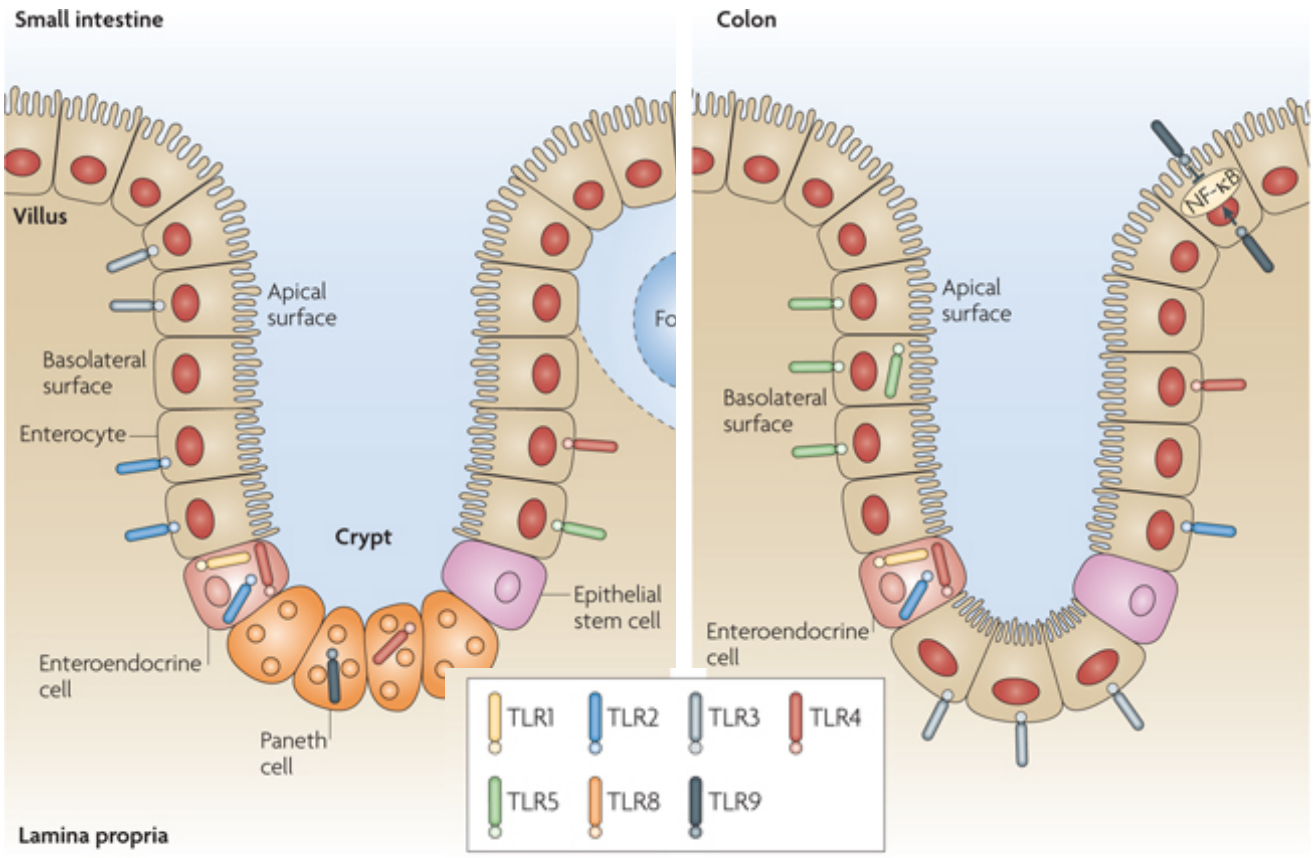


Figure 12: Toll-like receptor expression in the intestine. In the human small intestine the expression of TLR3, TLR4 and TLR5 has been shown on the basolateral surfaces of villus enterocytes. Enteroendocrine cells were found to express TLR1, TLR2 and TLR4, whereas their location on the apical or basolateral side is not clear. TLR9 and TLR4 are expressed in the cytoplasm of Paneth cells. In the human colon TLR3 and TLR5 are abundantly expressed on the basolateral side, whereas TLR2 and TLR4 expression is low. TLR9 is expressed apical as well as basolateral, with variable signaling outcome (adapted from [129]).

response by activation of transcription factors NF-κB and Jun N-terminal kinases (JNK) 1 or JNK2, and the secretion of IL-8 [132]. TLR4, which is the receptor for LPS from Gram-negative bacteria, was shown to be expressed at low protein levels in three different human intestinal epithelial cell lines. Moreover, they lacked accessory molecules necessary for activation, such as CD14 and MD2, which rendered them unable to recognize of LPS [133]. In a cell line derived from the mouse small intestine, TLR4 was found to be restricted intracellular to the Golgi apparatus [134,135]. Therefore activation of TLR4 requires the presence of cytosolic LPS, which could be provided by intracellular pathogens, but not commensal bacteria. The same is probably true for other classes of cytosolic innate immune receptors expressed by intestinal epithelial cells, such as Nod-like receptors (NLRs) and helicases.

Additionally, the TLR signaling pathways possess build-in negative regulators, which allow the shut down of an immune response and the introduction of tolerance. One member of the IRAK family, IRAK-M, is induced upon TLR stimulation and in return negatively regulates TLR signaling [137]. Signaling through IRAK proteins can also be inhibited by Toll-interacting protein (TOLLIP), which is a suppressor of TLR2 and TLR4 signaling [138]. IECs from patients with inflammatory bowel disease failed to up regulate Tollip expression, suggesting that this may contribute to chronic inflammation [139]. Other examples are single immunoglobulin IL1R-related molecule (SIGIRR), which is a negative regulator of TLR4 and TLR9 signaling [140] and A20, a zinc-finger protein, which has been shown to inhibit NF-κB [141]. A20-deficient mice develop severe intestinal inflammation, suggested that this gene is critical for limiting inflammation in the gut [142].

Besides the regulation of TLR expression and signaling, the host has other tricks to tolerate contact with MAMPs, such as for example LPS. Fernandez and colleagues could show a mechanism of LPS neutralization by transcytosing dimeric IgA [143]. The antibodies bind internalized LPS within the apical recycling endosome (ARE) in murine intestinal epithelial cells. Also the zebrafish possesses an enzymatic mechanism for LPS neutralization, the intestinal alkaline phosphatase (IAP). This enzyme is induced during establishment of the gut microbiota and localizes to the intestinal lumen brush border, where it detoxifies the endotoxin component of LPS [144].

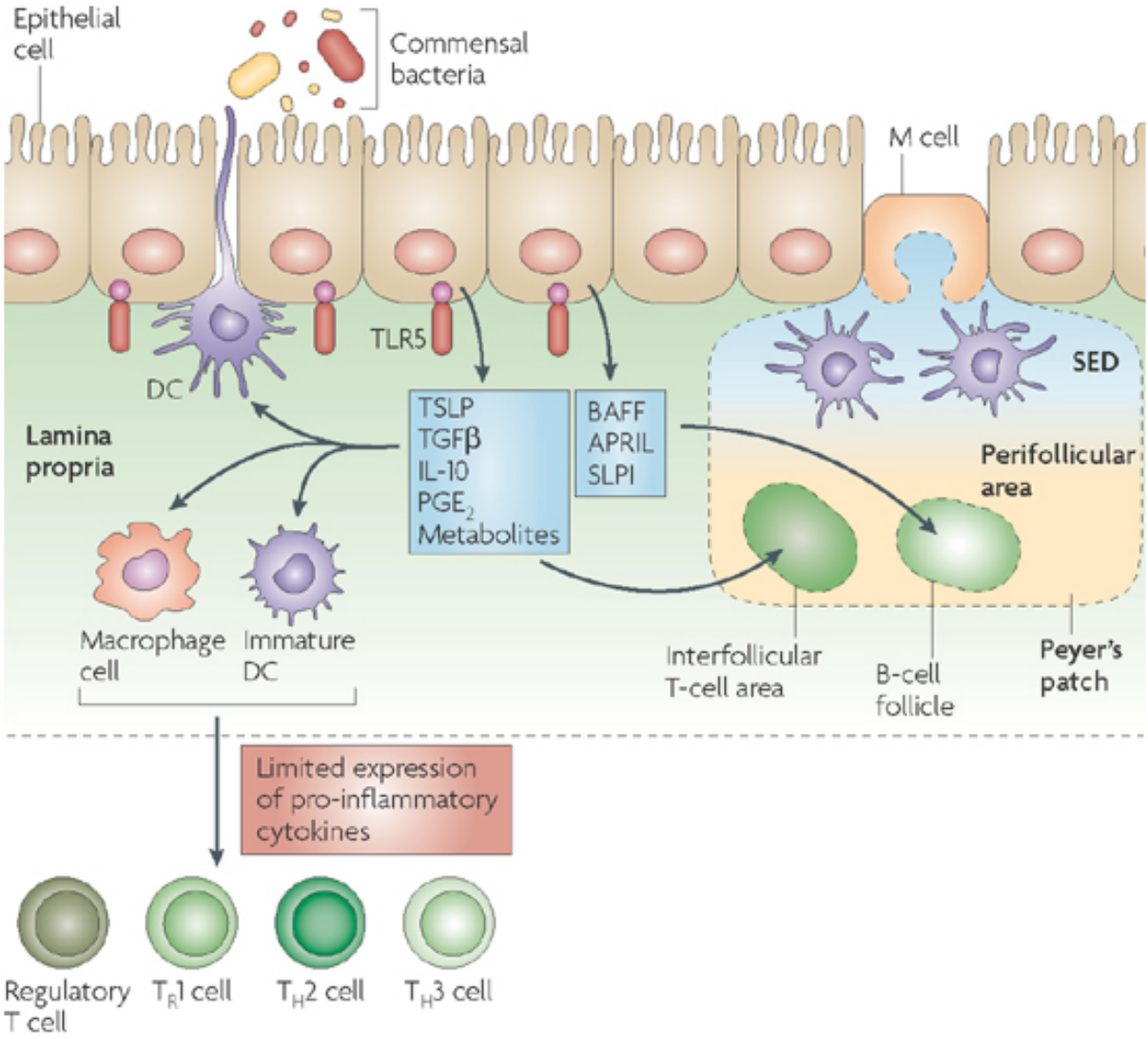


Figure 13: Intestinal epithelial cells secrete tolergenic factors. The gut microbiota stimulate IECs to secrete tolerogenic factors, such as thymic stromal lymphopoietin (TSLP), transforming growth factor-beta (TGFβ), prostaglandin E2 (PGE2) and interleukin-10 (IL10), which directly influences the expression of pro-inflammatory cytokines by dendritic cell (DC) and macrophages lamina propria, resulting in the predominance of T regulatory lymphocytes (Tregs). In addition IECs secrete B cell and IgA promoting factors, such as APRIL (a proliferation-inducing ligand), B-cell-activating factor (BAFF) and secretory leukocyte peptidase inhibitor (SLPI). Thereby, IECs communicate with immune cells in the intestinal microenvironment and regulate the functions of both antigen-presenting cells and lymphocytes (from [136]).

I.5.2.3 The intestinal epithelium as communicator with immune cells

Intestinal epithelial cells act as the interface between the microbiota and the gut associated immune system. They play an important role in the communication of signals, which help to maintain homeostasis and tolerance [145] (Figure 13). For example they secrete a variety of factors, such as thymic stromal lymphopoietin (TSLP), transforming growth factor beta (TGF-β), interleukin-10 (IL10), and prostaglandin E2 (PGE2), to suppress the production of proinflammatory cytokines and maintain a tolerogenic state of dendritic cells and macrophages in the lamina propria [146,147]. Furthermore, they produce mediators for maturation and proliferation of primed B cells in the gut associated lymphoid tissues, such as B cell activation factor (BAFF), proliferation-inducing ligand (APRIL) and SUN-like Protein (SLP1) [148].

I.5.3 Myeloid cells in intestinal tissues

Intestinal myeloid cells, such as DCs and macrophages, are important gatekeepers and defenders against invasion and infection. But in the maintenance of immune homeostasis they are instructed by the intestinal epithelium to maintain a state of tolerance (Figure 14). They can be divided into several subsets, possessing different abilities and performing variable tasks [149]. The major subsets of intestinal dendritic cells and macrophages involved in surveillance and tolerance are discussed below.

I.5.3.1 Intestinal dendritic cells

$CD11b^+CD103^+$ intestinal DCs constantly survey their microenvironment and coordinate the initiation and polarization of adaptive immune responses. Their hallmark activities are antigen uptake, migration and the capacity to prime T and B cells in gut-associated lymphoid tissue [150]. $CD103^+$ DCs play an important role as stimulators of Treg expansion via the production of TGF-β, retinoic acid [151,152], TSLP [153] and the tryptophan-catabolizing enzyme indoleamine 2,3 dioxygenase (IDO) [154]. IDO catalyzes the metabolism of tryptophan to metabolites such as kynurenine derivatives. This causes a depletion and tryptophan starvation for effector T cells and inhibits their proliferation, survival and activation [155]. Therefore IDO is able to act on two opposing T cell subtypes to support mucosal tolerance [156]. The main activity of $CD103^+$ cells is maintaining the tolerogenic state, but they can also act as potent T cell activators under inflammatory conditions, for example in models of experimental

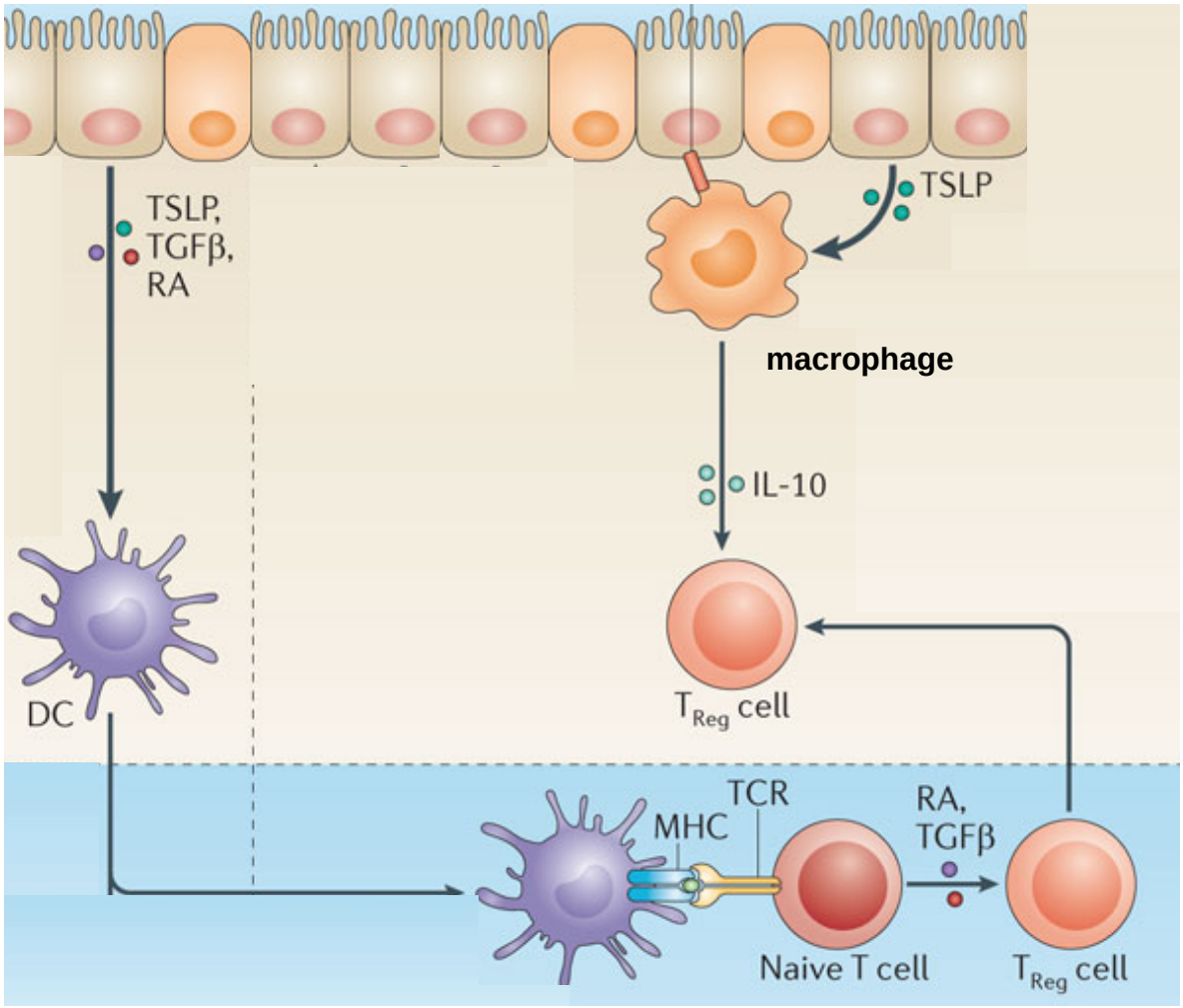


Figure 14: Tolerogenic dendritic cells and macrophages in the intestine. IECs condition DCs and macrophages towards a tolerogenic phenotype through the production of TSLP, TGFβ and retinoic acid (RA). These DCs promote the differentiation of naive CD4+ T cells into Treg cells. Expansion of converted Treg cells is induced by macrophages that are conditioned to produce IL10 by TSLP-mediated stimulation (adapted from [2]).

colitis [157]. They are able to stimulate $CD4^+$ and $CD8^+$ T-cell proliferation and induce cytotoxic T lymphocytes, therefore promoting the defense of the mucosa [158,159]. Following stimulation with flagellin, $CD103^+$ DCs were shown to induce Th17 differentiation [86]. Besides, they produced IL23, leading to induction of IL22 production in innate lymphoid cells, which in turn activates secretion of the mouse antimicrobial c-type lectin REG3γ (regenerating islet-derived protein 3 γ) by intestinal epithelial cells [160]. Thus $CD103^+$ dendritic cells play a double role in maintaining intestinal immune tolerance and promoting protective immunity.

I.5.3.2 Intestinal macrophages

Intestinal macrophages are the largest reservoir of body macrophages [162]. Lamina propria resident macrophages are educated by intestinal epithelial cells to show certain unresponsiveness towards MAMPs [163]. Macrophages isolated from normal human intestine lack CD14, a surface receptor involved in the response to LPS, and CD89, the receptor for IgA (FcalphaR). Therefore they show reduced LPS-induced cytokine production and LPS- and IgA-enhanced phagocytosis [164]. Their abilities to sample antigens and prime T cells complements the role of intestinal DCs, yet they are inferior to DCs in accomplishing those tasks. CX_3CR1^+ intestinal macrophages projected *trans*-epithelial dendrites (TEDs) extensions into the gut lumen to sense and potentially sample its bacterial content [150]. The capacity of CX_3CR1^+ cells to prime naive T cells might be less efficient compared to DCs because of their faster processing of cargo and the lack of expression of the chemokine receptor CCR7 that is required for migration to the MLNs [165]. CX_3CR1^+ macrophages were found to promote the differentiation of naïve $CD4^+$ T cells into $FoxP3^+$ Treg cells *in vitro* [166]. Moreover, they produce substantial amounts of IL10 in response to the microflora, which promotes the persistence of Foxp3 expression in Treg cells [167,168]. This highlights their role in maintenance of functional Treg cells that returned from the LNs to the tissue.

I.5.4 Regulatory T cells

Lamina propria $CD4^+$ and $CD8^+$ T cells provide a protective army of intestinal defense that limits spread of bacteria in case of an epithelial barrier breach. At the same time the activity of these effector cells presents an inherent risk of inappropriate inflammation, especially in the case of recognition of commensals or

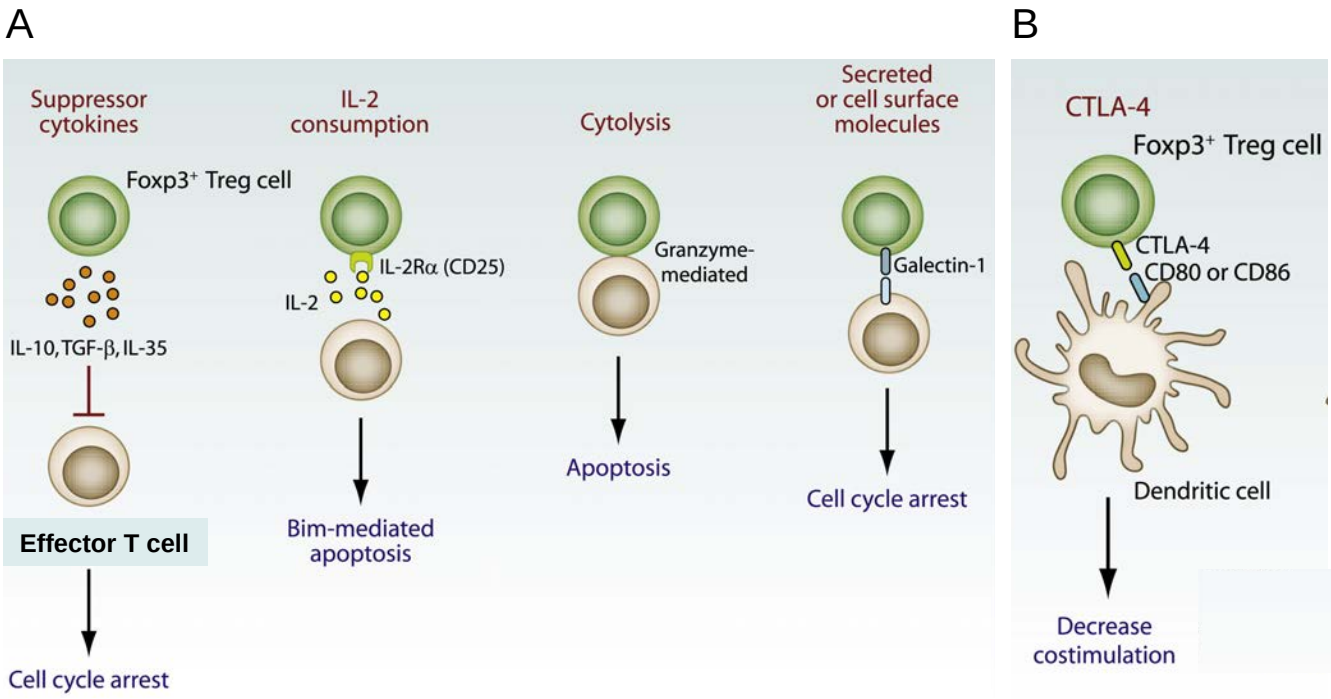


Figure 15: Inhibitory mechanism of regulatory T cells. (A) Treg cells may secrete suppressor cytokines, such as IL10, TGFβ and IL35, which inhibit the function of effector T cells. Moreover, Treg cells compete with effector T cells for IL2, resulting in IL2 deprivation of the effector cells and Bim-mediated apoptosis. Additionally, Treg cells may function as cytotoxic cells and directly kill effector cells in a granzyme-dependent manner. Activated Treg cells may express molecules on their cell surface (e.g., galectin-1), which can interact with receptors on effector T cells resulting in cell cycle arrest. (B) Tregs also exert inhibitory functions on APCs, for example via the expression of CTLA-4 on their surface, which mediates downregulation or prevents the upregulation of CD80 and CD86, the major costimulatory molecules on antigen-presenting cells (adapted from [161]).

food antigens. $CD4^+Foxp3^+$ Treg cells are important suppressors of lymphocyte-mediated intestinal inflammation [68]. As their main task is the inhibition of priming and differentiation of effector T cells, they also have to target the activity of antigen presenting cells. Studies *in vitro* have identified multiple mechanisms of Treg - mediated suppression, some of which will be discussed below [161] (Figure 15). Yet it is unclear if all these findings apply to *in vivo* situations.

IL2 is an important cytokine responsible for the development and differentiation of T cells into effector cells [169]. It is itself produced by T cells during an infection and functions in an autocrine manner [170]. Therefore IL2 is an important target for Tregs for the inhibition of effector T cells. Several studies have reported inhibition of IL2 mRNA induction by Tregs in effector T cells, and thereby prevention of the autocrine T cell activation by this cytokine [171,172]. Another mechanism to interfere with this process has been described as “IL2 consumption”. Treg cells possess the capacity to compete with effector T cells for IL2, consume it and thereby inhibit their proliferation. In a final step this resulted in apoptosis of the effector T cell, mediated by the proapoptotic factor Bim [173]. The secretion of anti-inflammatory cytokines, such as IL10, TGFβ and IL35 [174], by Treg cells can Additionally, inhibit the function of responder cells. Besides, they can induce ligand-receptor-mediated cell cycle arrest and apoptosis in effector T cells, for example via the beta-galactoside binding protein galectin-1 [175]. Furthermore, Treg cells were shown to express granzyme A and can directly kill effector cells in a perforin-dependent manner, similar to $CD8^+$ cytotoxic cells [176].

Some Treg cells employed mechanisms primarily affect the function of APCs. Studies showed that the Treg cell surface molecule cytotoxic T-lymphocyte antigen 4 (CTLA-4) can bind to costimulatory molecules CD80 and CD86 on the surface of DCs and induce their downregulation [177-179]. This limits the capacity of DCs to stimulate naïve T cells via CD28. Signaling via CTLA-4 and CD80/CD86 also induces DC expression of IDO, whose immunoregulatory mechanism have been described above. CTLA-4 seems to be of major importance in maintenance of homeostasis. Mice with defective CTLA-4 expression develop systemic autoimmunity shortly after birth [180]. Moreover, administration of anti-CTLA-4 antibody in an IBD model was found to counteract the ameliorating effects of Treg cell [181].

I.5.5 Other intestinal T lymphocytes

Intraepithelial lymphocytes (IEL) are a specialized subset of gut T cells located at the basolateral side of epithelial tight junctions [182]. The majority of IELs are $CD8^+$ and are further divided into two subgroups depending on the two chains that compose their T cell receptor: either alpha beta (αβ) or gamma delta (γδ) heterodimers. The microbiota are important regulators of IELs. Ismail *et al*. showed microbiota-dependent induction of IEL chemokines, proinflammatory cytokines, and REG3γ [183]. Moreover, γδ IELs play an important role as responders to bacteria or intestinal injury. On one hand they express proinflammatory cytokines and chemokines that recruit neutrophils, eosinophils, and T cells. On the other hand they promote epithelial healing via production of keratinocyte growth factor, which stimulates epithelial cell proliferation and restoration of barrier function [184].

Furthermore, the lamina propria and GALT are home to different subsets of $CD4^+$ Th cells [185]. They all possess distinct cytokine profiles and fulfill different requirements in the adaptive immune system of the gut [186]. Th1 cells for example, produce interferon γ, TNFα, and IL12 and are important for the defense against intracellular pathogens. Th2 cells produce IL10, IL13, IL5, and IL4 and play a role in the defense against helminthes. Th17 cells produce IL17, IL21, and IL22 and provide protection against extracellular bacteria and parasites. In general Th activation can result in activation and proliferation of cytotoxic T cells, activation of myeloid cells and B cell differentiation and antibody production. To maintain a healthy gut environment the presence of Treg cells and T effector cells needs to be balanced. Disturbance of this balance can have negative influences on the gut microbiota as well as on the host.

I.5.6 Bacterial strategies to maintain tolerance

Besides, the hosts desire to keep the immunological homeostasis in the gut, we must not forget the other side of the story. Naturally it is also in the highest interest of the symbiotic bacteria colonizing our gut to maintain their living environment and stay undetected from their hosts immune system. Therefore some of them harbor mechanism to temper with and control the immune response of their host. For example various strategies to manipulate the TLR downstream signal transduction through NF-κB have been identified. *Bacteroides thetaiotaomicron* is able to

suppress NF-κB function by enhancing the nuclear export of its subunit p65 through a PPAR-γ-dependent pathway [187]. Nonpathogenic Salmonella strains inhibit polyubiquitination and following degradation of the NF-κB inhibitor IκB-α, thereby preventing subsequent activation and nuclear translocation of the transcription factor [188]. *Lactobacillus casei* induces down regulation of several components of the proteasome, thereby protecting IκB-α degradation and NF-κB activation [189]. Additionally, commensals possess physical features that mark them as harmless. Generally they lack virulence factors, such as adhesins and invasins. Moreover, some species possess altered MAMPs. Bacteroidetes species for example carry pentacetylated lipid A on their LPS, which is only poorly recognized by TLR4 [190]. Also *Helicobacter pylori* flagellin is only mildly reactive for TLR5 in human gastric epithelial cells [191]. A similar flagellin structures could be imagined for gut commensal species.

Summary

In chapter I, we have discussed about the physiology of the human gut, the role of the gut microbiota in various aspects of development and health of the host, and how we maintain a peaceful and beneficial coexistence with the trillions of microbes inhabiting our body. It has become clear that the communication between the microbiota and the host is vital to develop and maintain a healthy organism. But also, we must not forget that this beneficial coexistence is fragile and that the maintenance of balance is regulated in a highly complex manner. In this regard, in completion of chapter I.4.3 “Development and activity of the gut immune system”, it has to be mentioned that the gut microbiota induce the expression of a broad spectrum of AMPs in the intestine. This part of the dialogue between microbes and host is crucial for the maintenance of homeostasis, as it controls the direct contact of bacteria with intestinal epithelial cells. As chapter II focuses exclusively on AMPs, their expression and characteristics, as well as their role in the bodies immune response, but also their association with human pathologies will be discussed in great detail. Furthermore, the capacities of microbes to evade the destructive effects of AMPs will be described. Finally, we will review different inducers and repressors of AMPs gene expression, in order to approach the question of how these genes are regulated in an inducible manner.

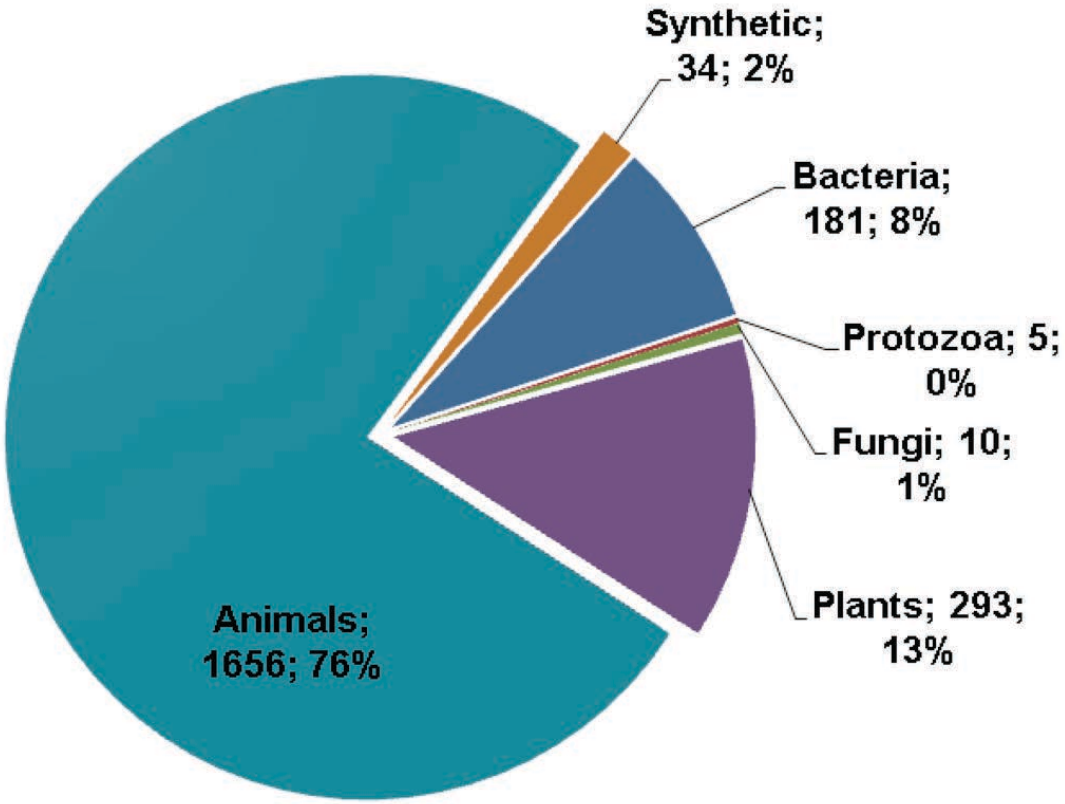


Figure 16: Antimicrobial peptides noted in the Antimicrobial Peptide Database (APD). This graph shows the percentage of antimicrobial peptides coming from different sources and noted in the APD as of 2013 [192].

Chapter II – Antimicrobial peptides

AMPs are evolutionary conserved intrinsic antibiotics and an important part of our innate immune system. The antimicrobial capacities of tissue, body fluids and secretions were first described in the end of the 19th century. Many researchers described the antibacterial activity of molecules in leucocyte extracts, blood, saliva and tears against Gram-positive and Gram-negative bacteria [193]. Already then they were recognized for their ability to slow bacterial growth or kill invading microorganisms through interaction with the negatively charged cell surface. Moreover, they were thought to aid both the innate and the adaptive immunity. In the following decades hundreds of different molecules have been identified, isolated and described in prokaryotes, plants, invertebrates and animal species. To date, the Antimicrobial Peptide Database (APD, http://aps.unmc.edu/AP/main.php) of the University of Nebraska Medical Center contains 2349 antimicrobial peptides (214 bacteriocins, 305 plant AMPs, 12 fungal AMPs, and 1771 animal host defense peptides) [194] (Figure 16).

II.1 Antimicrobial peptides throughout the kingdoms of life

In order to survive and flourish in a microbe-dominated environment, plants and animals developed an array of antimicrobial molecules, potent to fight bacteria, viruses, fungi and protozoa [195]. Even though AMPs are evolutionary conserved, the sequence diversity in between species is remarkable, possibly reflecting the adaptation to unique microbial environments and food sources. Despite the huge diversity all AMPs share certain traits and structural characteristic. Most of them are relatively short (12-50 amino acids) (Figure 17 A), derived from larger precursors and can undergo post-translational modifications including proteolytic processing, glycosylation or cyclization. Besides their diversity of sequences they all possess an amphipathic structure with sections of hydrophobic and cationic amino acids (Figure 17 C, D) and carry a positive charge (Figure 17 B). According to their amino acid composition and conformational structures AMPs can be divided into several classes: for example α-helical peptides (eg. LL37), β-sheet structures with disulfide bonds

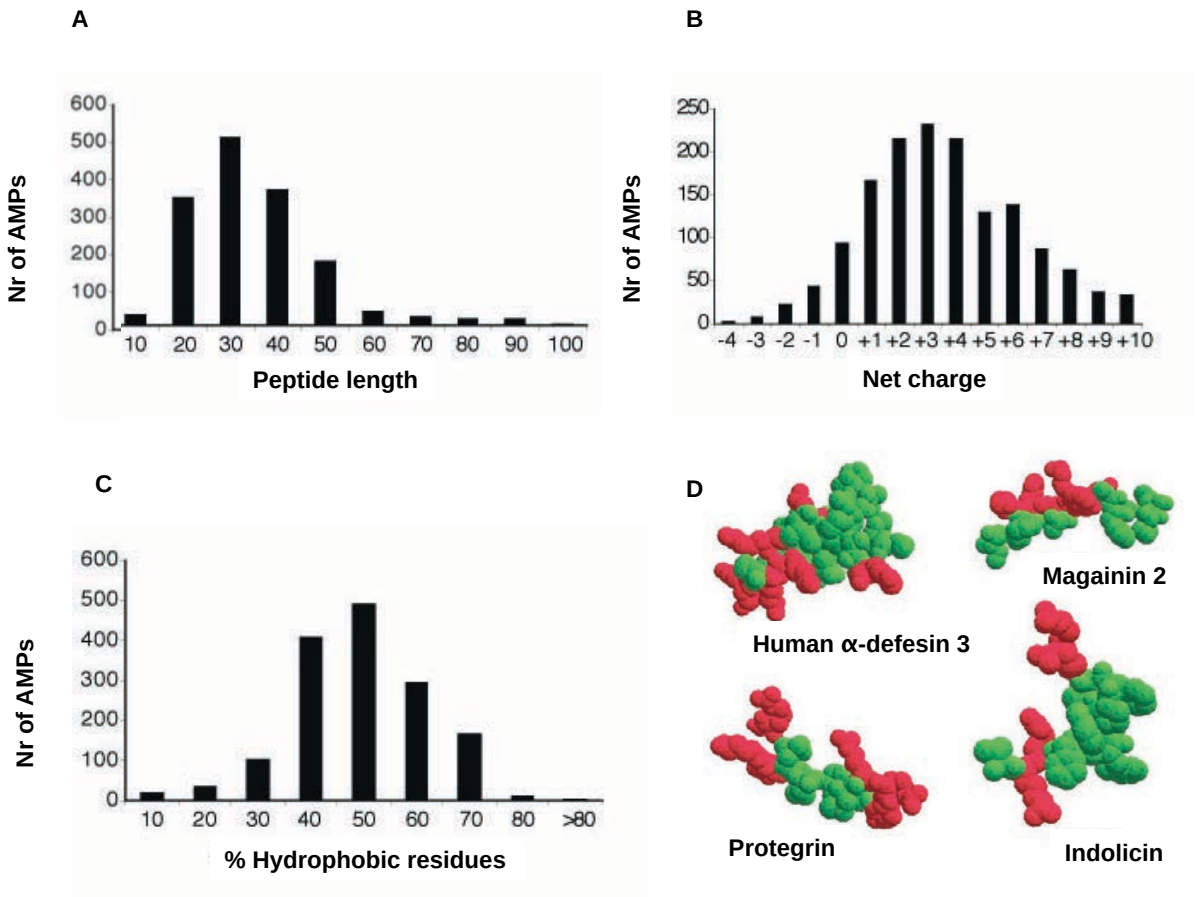


Figure 17: Diversity of AMP characteristics. Most AMPs have a short length of amino acids (A) and carry a positive charge (B). The majority of them possess an amphipathic structure with a cluster of hydrophilic cationic (red) and hydrophobic (green) residues (C,D) (adapted from http://aps.unmc.edu/AP/facts.php, [195])

(eg. β-defensins), extended (eg. indolicidin) or loop structures (eg. cyclic θ−defensin) [196] (Figure 18).

In both animals and plants, the innate immune response is triggered after recognition of conserved MAMPs by PRRs on multiple cell types [197,198]. The conserved production of AMPs after contact with microbes throughout all kingdoms of life reflects the ancient origin and the importance of this type of defense response [195]. Expression patterns differ between tissues and cell types, but usually AMPs are expressed as a cocktail of different molecules from different classes. In animals, AMPs are produced by epithelial cells and secreted onto the surface for the protection of the host's borders with the external microbial world. Additionally, immune cells can produce AMPs and store them in granules, from which they are released upon microbial contact. But in some cases they can also be secreted into circulating fluids (e.g. the bloodstream or the hemolymph), which deliver AMPs to infection sites. In plants, AMPs are probably not circulating, but are either constitutively expressed in specific sensitive organs or are induced by microbes at the site of infection and also systemically [199]. The presence and expression of AMPs in different phyla will be discussed in the next chapters on hand of certain examples of species.

II.1.1 AMPs in plants (eg. *Arabidopsis thaliana)*

The plant immune system employs various classes of AMPs including defensins, thionins, lipid-transfer proteins, hevein- and knottin-like peptides, and macrocyclic peptides as well as secondary metabolites, proteins and reactive oxygen species (ROS) with antimicrobial activity [197,200]. Expression of these molecules is upregulated in a receptor-mediated manner, after sensing of plant danger signals upon tissue damage or pathogen associated patterns. *In vitro* studies have uncovered their involvement in biotic and abiotic stress response. Thionins possess high inhibitory activity against fungi and bacteria including human pathogens, such as *Candida albicans*. Additionally, they are toxic to mammalian, insect, and plant cells. Knottin-like and macrocyclic AMPs exhibit insecticidal, antimicrobial, anti-human immunodeficiency virus (HIV) and hemolytic activities. Interestingly, plant AMPs have also been found to be involved in processes other than immunity. They mediate

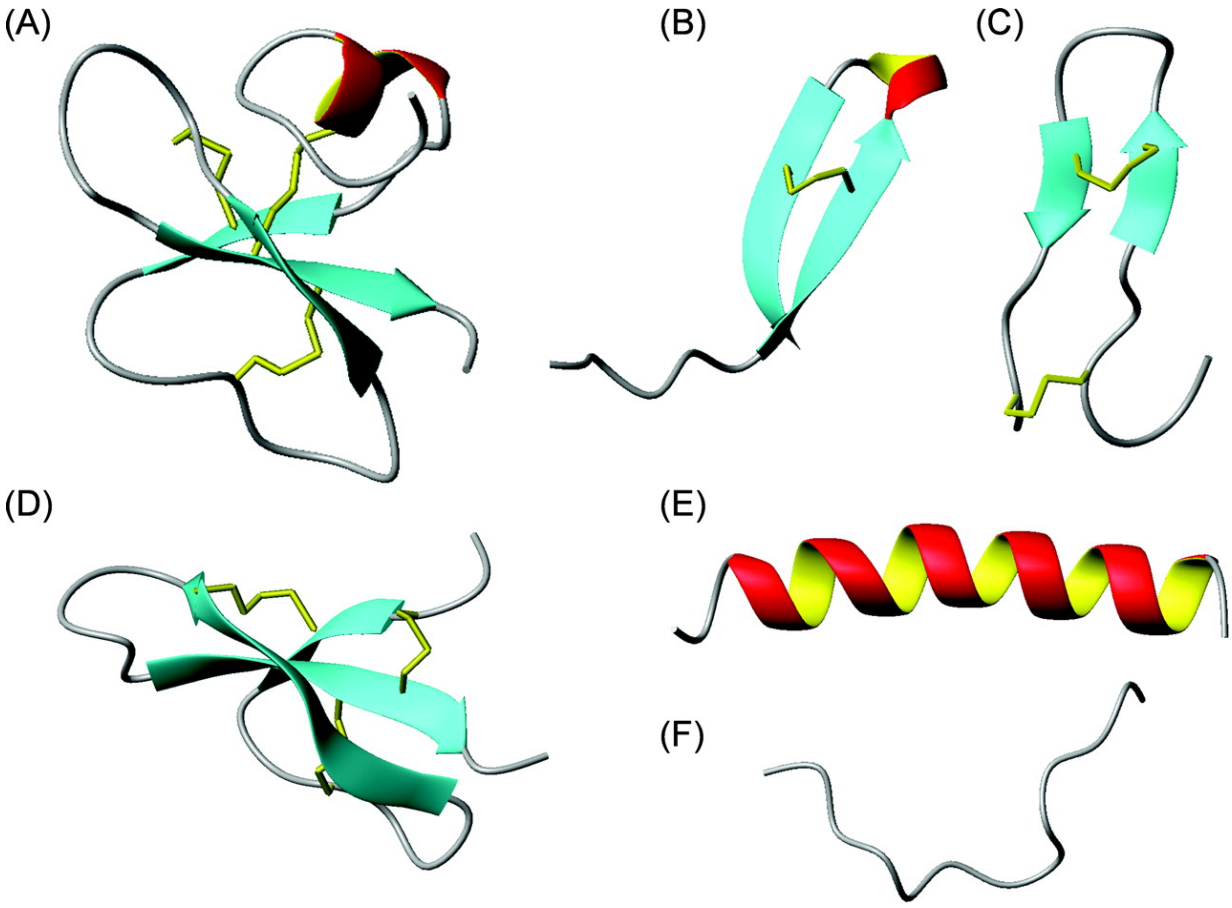


Figure 18: Examples for possible AMP protein structures. AMPs can be divided into multiple structural classes. Human β-defensin-2 for example possesses a mixed structure of a short α-helix and the characteristic β-sheets linked by disulfide bonds (indicated in yellow) (A). Furthermore, we can find looped structures, for example in the insect AMP thanatin (B), pure β-sheeted peptides, such as polyphemusin from the horseshoe crab (C) and the rabbit kidney defensin-1 (D), pure α-helical structures such as LL37 and magainin-2 (E) and extended structures, such as bovine indolicidin (F) (from [201]).

complex signaling processes between male and female gametophytes [202] and control the density of pores in the leaves for gas exchange [203].

Plant defensins (PDFs) are the largest and best-studied class of AMPs and wide spread throughout the plant kingdom [204]. More than 300 putative defensin-like genes were discovered in the model plant *Arabidopsis thaliana*, which is widely used to study their expression and modes of action [205,206]. Most of them form clusters suggesting common origin by successive gene duplication events. Each gene consists of two exons and a single intron in the signal peptide domain. They are small (5kDa), basic peptides with a characteristic three-dimensional folding pattern that is stabilized by eight disulfide-linked cysteines. PDFs are expressed in seeds, stems, roots, leaves and floral organs. Expression of PDFs can be constitutive, for example in sensitive tissues such as seeds, developmentally regulated or induced by different abiotic and biotic stress factors, including cold, drought, heavy metals or microbial pathogens [205]. They are capable to inhibit growth of a broad range of fungi and yeasts *in vitro* [207,208] and some of them are active against bacteria [209]. A study by DeConinck *et al.* showed that constitutive overexpression of the PDF AtPDF1.1 in *A. thaliana* showed reduction in symptoms caused by the pathogen *Cercospora beticola* [210].

II.1.2 AMPs in bacteria

Even some bacteria and archea produce a variety of defense peptides, the so-called bacteriocins [211]. These molecules are toxic for bacteria of the same species or different genera and are used in combat against competitors in an ecological niche. Protection against self-destruction is conveyed by the expression of immunity proteins. The first bacteriocins, the *E. coli* produced *colicine,* was discovered in 1925 by Gratia. Best described today are the lanthionin-containing antibiotics “lantibiotics”, produced by lactic acid bacteria. The structure of bacteriocins and modes of bacterial killing are highly similar to described AMPs in other phyla. Moreover, these peptides can be applied to a wide commercial use. The first discovered lantibiotic Nisin A for example is used as a food preservative in over 50 countries [212]. But their application could also be of use to medicine. Bacteriocins from *Staphylococcus* for example were shown to be active against multi-drug resistant *S. aureus* [213].

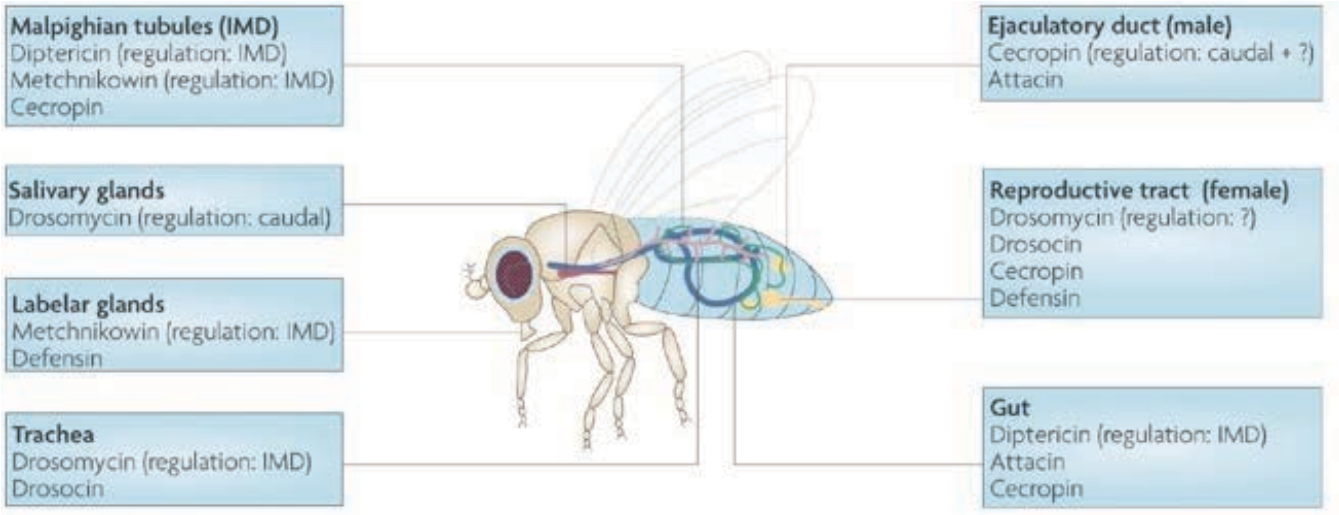


Figure 19: Expression of AMPs in *Drosophila melangoster*. The figure illustrates the expression of AMPs at various body sites of *Drosophila*. Some AMPs are under the transcriptional regulation of the homeobox gene product Caudal, which is responsible for the constitutive local expression of antimicrobial peptides cecropin and drosomycin in a tissue-specific manner, for example in the reproductive tract or salivary glands. Others are induced upon microbial contact as indicated via the Toll or Imd pathway.

II.1.3 AMPs in insects (eg. *Drosophila melangoster*)

The genome of the common fruit fly *Drosophila melangoster* encodes 20 AMP genes, which can be characterized into 7 different classes [214]. The expression of these genes is dependent on MAMP recognition by PRRs of the Toll or immune deficiency (Imd) pathway [198]. Mutants in PRR pathways do not express AMPs and were shown to be highly susceptible to systemic infections [215,216]. The conservation of signaling pathways for the activation of AMPs, such as the Toll pathway, shows the ancient origin of this defense mechanism in metazoan evolution and strengthens the use of *Drosophila* as a potent model to decipher general innate immune mechanisms in animals. Upon induction, AMPs are expressed in epithelia such as those beneath the cuticle, in the alimentary tract, and in tracheae [198,217] (Figure 19). A specialty of *Drosophila* is the expression of AMPs in the fat body, which is the equivalent of the liver and a major immune-responsive tissue. Here, AMPs are secreted into the hemolymph circulatory system, where they readily reach their effective concentrations. Some AMPs are very stable due to intramolecular disulfide bonds and can still be detected in the hemolymph several weeks after challenge [218]. *Drosophila* AMPs are small (<10 kDa), with the exception of the 25 kDa Attacin, cationic, membrane-active and possess activity against Gram-negative and Gram-positive bacteria and fungi [219].

II.1.3 AMPs in animals (eg. *Mus musculus*)

AMPs are widely conserved and homologues of certain families can be found in different mammalian species. This allows for the use of animal models to study the role of AMPs in health and disease. The mouse is a commonly used model to study cathelicidin and defensin expression. Nevertheless, the transferability of results obtained in mice to humans is not guaranteed. Mouse intestinal Paneth cells express a defensin family called cryptdins [220-222]. Sequencing of cryptdin cDNAs demonstrated the presence of at least 17 different mRNAs, identifying cryptdins as the largest known defensin family. Six characterized cryptdin genes all have very high overall nucleotide similarity (85%), suggesting gene duplication events that occurred relatively recently in evolution. Furthermore, a recent study identified a family of cryptdin-related sequence peptides, which exist in the form of covalent dimers in mouse intestinal tissue [224]. Interestingly, the mouse lacks myeloid

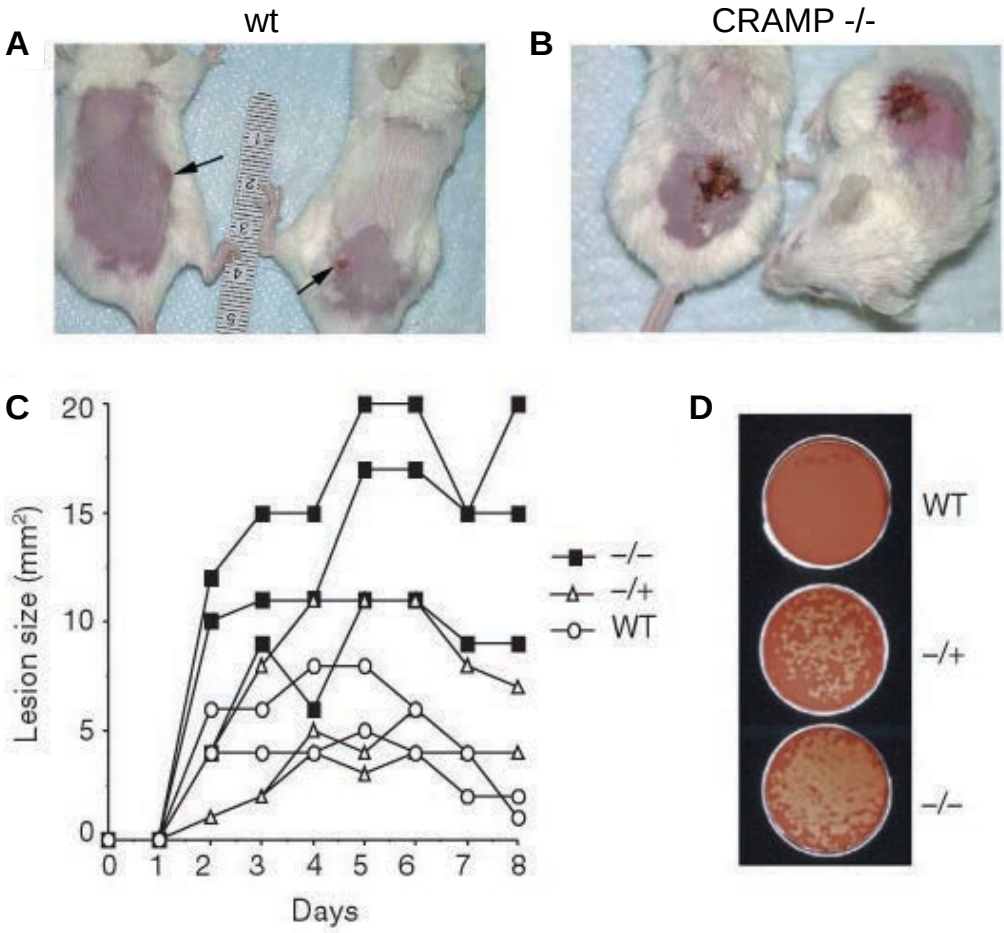


Figure 20: CRAMP deficient mice are more susceptible to group A *Streptococcus* infection. CRAMP -/- mice show increased susceptibility upon subcutaneous inoculation with GAS (B) as compared to wild-type (wt) mice (A). Moreover, they show higher severity of lesions as measured in the area of necrotic ulcer in individual WT (circle), CRAMP +/- (triangle), and CRAMP -/- (square) mice shown against days after infection (C). Furthermore, the bacterial load in the tissue of CRAMP deficient mice is much higher as seen in GAS bacteria cultured from tissue biopsies of WT, CRAMP -/+ and CRAMP -/- mice (D) (from [223]).

defensins and therefore the peptide-based antibacterial defense by recruited neutrophils is limited [225]. Several mouse beta defensins have been identified in epithelial tissues, such as mouse β-defensin 1 (mBD-1), which is a constitutively expressed homolog to human β-defensin 1 (DEFB1) [226,227] [228], mouse β-defensin 2 (mBD-2) [229] and mouse β-defensin 3 (mBD-3), the murine homologue of human β-defensin 2 (DEFB2) [230]. The mouse also possesses a cathelicidin gene encoding the cathelin-related antimicrobial peptide (CRAMP), which is expressed at various body sites, such as the intestine, skin and urinary tract [231].

The mouse is a competent model to show effectiveness of AMPs in the fight against pathogens *in vivo*. Mice deficient in mBD-1 were not able to achieve clearance of *Haemophilus influenzae* from lungs or *Staphylococcus* sp. from the urinary tract [232,233]. Similarly, inactivation of the mouse cathelicidin gene increased their susceptibility to GAS subcutaneous infection [223] (Figure 20). In another study CRAMP-deficient mice could not maintain sterility of the urinary tract [234].

Finally, genetically manipulated mice are a useful tool to study the regulation and expression of AMPs. A prominent example is the investigation of inflammatory bowel diseases, which is associated with mutations in several susceptibility genes and following decreased expression of defensins in the intestine, as will be discussed later in this chapter [235].

II.2 AMPs throughout the human body

Each interface of the human body has its characteristic repertoire of different AMPs. Skin keratinocytes and mucosal epithelial cells produce constant low levels of AMPs, which can be drastically increased upon certain stimuli such as infection and injury (chapter II.7). Furthermore, tissue resident macrophages, dendritic cells, mast cells and recruited neutrophils are capable of backing up the epithelial response with their own AMP repertoire. Key antimicrobial peptides are cathelicidin and the defensins, which will be described in greater depth in the scope of the presented thesis (chapter II.2.1 and II.2.2 respectively). The discussion of all AMP families expressed in the human body is way beyond the scope of this thesis.

Family	Representative proteins	Mechanism of action	Tissue sites of expression	Cellular sites of expression	Target organisms
α-defensins (cryptdins in mice)	DEFA5, DEFA6 (in humans); cryptdins 1–16 (in mice)	Membrane disruption	Small intestine	Paneth cells, neutrophils, macrophages	Gram-positive bacteria, Gram-negative bacteria, fungi, viruses, protozoa
β-defensins	BD1, BD2, BD3	Membrane disruption; lipid II binding (BD3)	Large intestine, skin, respiratory tract	Enterocytes, keratinocytes, respiratory tract epithelial cells	Gram-positive bacteria, Gram-negative bacteria, fungi, viruses, protozoa
Calprotectin (S100A8–S100A9)	NA	Metal chelation	Abscesses	Neutrophils	*Staphylococcus aureus*
Cathelicidins	LL37 (in humans); CRAMP (in mice)	Membrane disruption	Large intestine, skin, lung, urinary tract	Neutrophils, mast cells, epithelial cells	Gram-positive bacteria, Gram-negative bacteria, viruses, fungi
C-type lectins	HIP/PAP (in humans); REG3γ (in mice)	Peptidoglycan recognition; killing mechanism unknown	Small intestine	Epithelial cells (Paneth cells, enterocytes)	Gram-positive bacteria
Galectins	GAL4, GAL8	Unknown	Intestine	Broad expression, including by epithelial cells	Bacteria bearing blood group antigens
Lipocalin	Lipocalin 2	Sequestration of iron-laden siderophores	Broad expression, including intestine and lung	Macrophages, epithelial cells	*Escherichia coli*
Lysozyme	NA	Enzymatic attack on bacterial cell wall peptidoglycan	Intestine, eye, and more; secretions, including tears, saliva	Intestinal Paneth cells	Gram-positive bacteria; some activity against Gram-negative bacteria
Peptidoglycan recognition proteins	PGLYRP1–4 in mammals	Activation of bacterial two-component systems; PGLYRP2 is an amidase that targets peptidoglycan	Liver, intestine, skin, mammary gland	Epithelial cells	Gram-positive and Gram-negative bacteria
Phospholipase A2	NA	Hydrolysis of bacterial membrane phospholipids	Intestine; secretions, including tears, inflammatory fluids	Paneth cells, macrophages	Gram-positive bacteria
RNases	ANG4, RNase7	Unknown	Skin, intestine	Epithelial cells	Gram-positive and Gram-negative bacteria

Table 1: Antimicrobial peptides expressed in the human intestine. Epithelial and Paneth cells in the intestine express a wide variety of AMPs with multiple functions and killing activity against a broad range of microbes. NA, not applicable. (adapted from [236]).

Nonetheless, I want to briefly mention some important AMP families. For example the S100 proteins, with psoriasin as the most prominent member and one of the major AMPs expressed in the skin [237]. The S100 proteins represent a family of small, acidic proteins of 10–12 kDa containing two distinct EF-hand Calcium binding motifs [238]. Their expression was shown in the colon, keratinocytes and airway epithelium and they fulfill various intra- and extracellular functions. Killing activity has been shown against multiple pathogens, such as *Escherichia coli*, *Klebsiella*, *Candida* and *Staphyococcus* species, but the mechanism has not been elucidated [239,240]. Another class of AMPs are the elastase inhibitors, such as elafin and secretory leukocyte peptidase inhibitor (SLPI). They are present in granules of neutrophils, mast cells and macrophages and are also produced by a variety of epithelial cells. After secretion they are associated with extracellular matrix components and can kill Gram-positive and Gram-negative bacteria and fungi via membrane permeabilization [241]. Furthermore, there exist the peptidoglycan recognition proteins (PGLYRPs) [242]. Mammals possess 4 genes of PGLYRs and they are widely distributed in leukocytes and epithelial cells. They can kill a variety of pathogens by amidase-mediated peptidoglycan digestion and osmotic lysis. Moreover, c-type lectins represent a large family of receptors carrying carbohydrate recognition domains of the structural type “C”. C-type lectins comprise the collectins, the REG proteins [243]. Within the REG family we find REG3α, REG3β and REG3γ in the Paneth cells of the mouse small intestine and the hepatointestinal pancreatic/pancreatitis-associated protein (HIP/PAP) in Paneth cells of humans [244,245]. C-type lectins, such as REG3γ in the mouse, are important players in the maintenance of homeostasis by keeping the microbiota at bay and in distance to the intestinal epithelium [246]. Furthermore, the expression of C-type lectins was shown to be induced in the mouse intestine during pathogen infections or inflammatory conditions [247]. They possess antibacterial activity specific to Gram-positive bacteria, as they can bind to peptidoglycan in the bacterial cell wall [248]. Several AMPs expressed in the human body belong to the functional group of iron metabolisms proteins: Lactoferrin, hepcidin and lipocalin-2 exert their antimicrobial function via the sequestration of iron and membrane permeabilization [250-252]. The major AMP families expressed in the intestine are summarized in Table 1 to provide an overview of their expression, mechanisms and target organisms (Table 1).

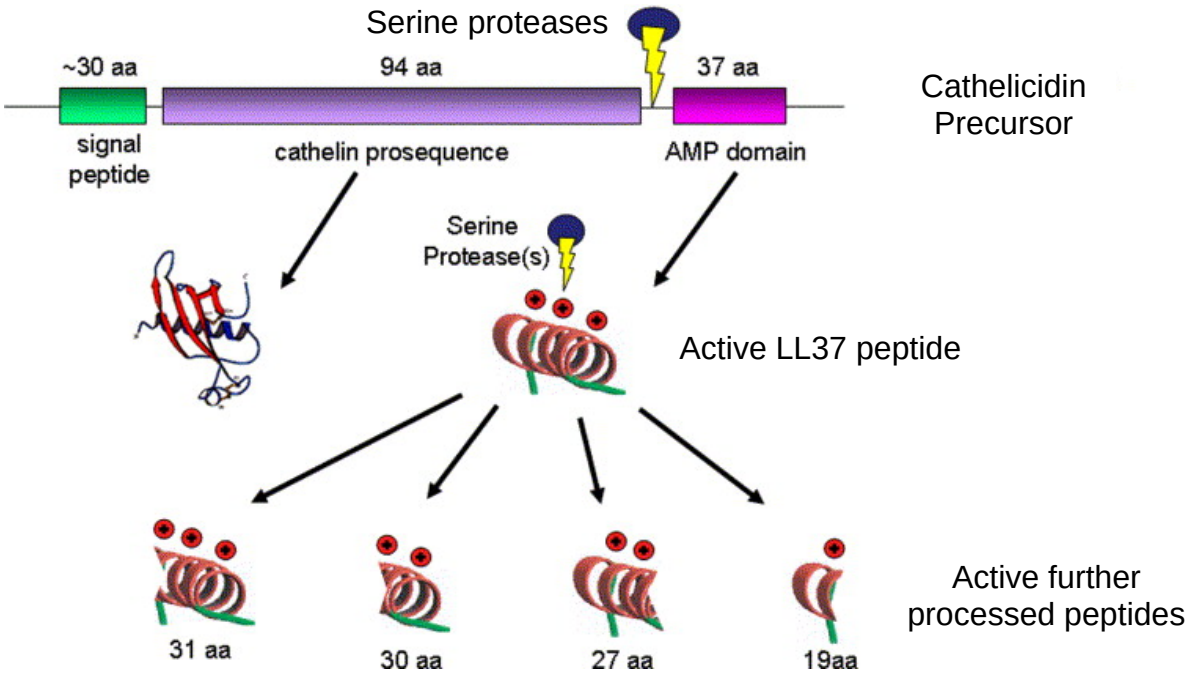


Figure 21: Processing of cathelicidin. Cathelicidin is synthesized as an inactive precursor protein. It consists of an amino-terminal signal peptide (green), a central cathelin domain (purple), and the characteristic 37 amino acid long inactive carboxy-terminal AMP domain (pink). Several serine proteases have been described to cleave the AMP domain to generate active LL37, as well as additional active peptides by further cleavage of LL37 (adapted from [249]).

II.2.1 Cathelicidins

The cathelicidins have been described in both invertebrate and vertebrate species, including humans [253]. In humans one single cathelicidin gene on chromosome 3p21.3 (CAMP) encodes the inactive 170 amino acid long precursor protein human cationic antibacterial protein of 18 kDa (hCAP18) [254]. The active mature peptide LL37, named for the two leucines that comprise its first two N-terminal residues and its length of 37 amino acids, is released from the C-terminus of hCAP18 by protease processing [255]. Additionally, LL37 secreted onto the skin surface can be further processed into smaller peptides, such as RK-30 and KS-30, which show even higher antimicrobial activity, but are less immunogenic [256] (Figure 21).

LL37 is expressed and stored in the granules of myeloid cells, neutrophils, mast cells and monocytes [254,257]. Additionally, it can be secreted by epithelial cells of the colon, urinary and respiratory tract and keratinocytes of the skin [258-261]. The active peptides generated by cathelicidin genes between species show little similarity to each other and are referred to as a group solely based on the highly conserved N-terminal region of the precursor protein known as the cathelin domain [253]. Some of these peptides can assume an α-helical conformation; others contain one or two disulfide bonds. Moreover, they vary in the enrichment of amino acids, such as prolin, arginine and tryptophane. They possess a broad range of microbial killing activity and also exert additional functions related to host defense, which will be discussed in more extent in following chapters. LL37 has been shown to neutralize LPS. Furthermore, it is chemotactic for neutrophils, monocytes, mast cells and T cells. It is also able to promote wound healing by stimulation of vascularization and re-epithelialization [262]. LL37 exerts important antibacterial action against multiple pathogens, for example in the skin against GAS [263]. In the intestine, LL37 was described to decrease the bacterial load of *Shigella* in a rabbit experimental model [264].

II.2.2 Defensins

Defensins are small (2-5 kDa), cysteine-rich cationic AMPs reported in invertebrates, vertebrates and even plants [266]. Mammalian defensins can be subdivided into α-, β- and θ-defensins [265] (Figure 22). All defensins contain six highly conserved cysteine residues, which form three pairs of intramolecular disulfide bonds. Thereby, they

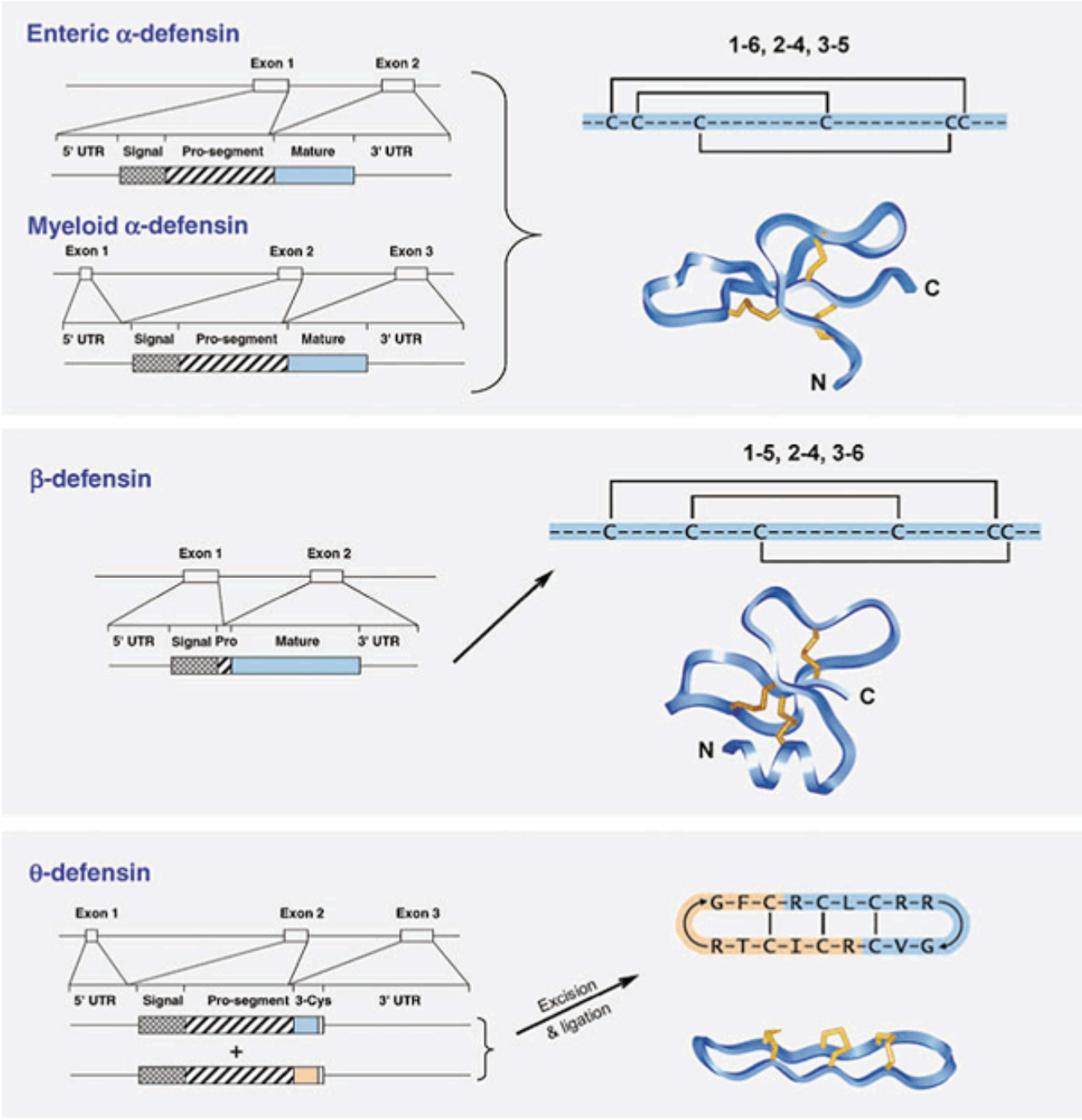


Figure 22: Defensin genes and peptides. The left side of the graph shows a representation of α-, β-, and θ-defensin encoding genes. Below the genetic structure the resulting transcribed proteins are depicted, indicating the signal sequence (checked), pro-peptide (striped) and the mature processed peptide (blue, yellow). On the right side of the graph cysteine-bonds are indicated in the mature peptide and the three-dimensional structure is depicted [265].

establish their beta-sheet structure. These bonds show specific differences between subgroups of defensins and allow their classification. All defensins are translated as inactive precursor proteins and activated by proteolytic processing. After secretion, they fulfill various tasks in the immune defense and regulation of homeostasis, which will be discussed in greater depth in the following chapters.

II.2.2.1 Alpha-defensins

The α-defensins are between 29 and 35 amino acids long. Their cysteines are linked in a 1–6, 2–4 and 3–5 pattern. Six α-defensins have been identified in humans. At first, they were discovered in granules from neutrophils and therefore named human neutrophil peptides (HNP) 1-4 [267]. Neutrophil α-defensins exhibit potent antiviral capacities. HNP-1, HNP-2, and HNP-3 show *in vitro* activity against adenovirus, papiloma virus, herpes virus, influenza virus, and cytomegalovirus, while HNP-4 inhibits HIV infection *in vitro* [268]. Later, two additional α-defensins (HD5 and HD6) were discovered in the Paneth cells of the small intestine, where they are stored in intracellular vesicles and released upon stimulation, for example by MAMPs [269]. Additionally, they were found in epithelial cells from the female urogenital tract [270]. HD5 exposes effective killing activity against various bacteria [271] as well as parasites [272]. HD6 showed only weak killing activity in *in vitro* assays, but recent work discovered a different mechanism of action that prevents even translocation of *Salmonella* in a transgenic mouse model [273]. In this study HD6 was found to polymerize and undergo ordered self-assembly into nanonets. These structures form *in vivo* and are able to entrap bacteria in the lumen. Both HD5 and HD6 possess antiviral capacities against various pathogenic viruses, such as herpes simplex virus, human papillomavirus, adenovirus, and influenza A virus [274-277]. Binding of the virus and inhibition of viral adhesion and entry to cells exert antiviral activity.

II.2.2.2 Beta-defensins

The characteristic disulfide bonds in β-defensins are located in a 1–5, 2–4 and 3–6 pattern. In humans six beta defensins with a length between 36 and 42 aminoacids have been identified. DEFB 1-6 are all expressed in epithelial cells of different body sites. DEFB1 is constitutively expressed while the others are inducible by a variety of stimuli as will be discussed later. We can find expression of DEFB1 throughout the gastrointestinal, respiratory, urinary and female genital tract [278-280] and to a lesser

extend in the pancreas and liver [281]. DEFB2 is expressed in the colon, skin and urinary and respiratory tract [280-282]. DEFB3 is expressed in epithelia of the skin and colon and in high concentrations in the saliva and cervicovaginal fluids [283]. DEFB4 expression was detected in testicles, stomach and the uterus [284]. DEFB5 and DEFB6 are specially found in the epididymis [285]. DEFB2 has shown antimicrobial activity *in vitro* against *Pseudomonas aeruginosa* and *E. coli* [286]. DEFB3 has potent antibacterial activity against *S. aureus* [287]. DEFB1, DEFB2, and DEFB3 possess also antifungal activity against multiple *Candida* species [288]. Some defensins showed only weak killing activity in *in vitro* assays. Recent work by Schroeder *et al.* discovered the necessity for biochemical activation in a reducing environment, for example in the case of DEFB1 [289].

II.2.2.4 Teta-defensins

The circular θ-defensins have only been described in leukocytes of the rhesus macaque and the olive baboon [290,291]. They are formed by two hemi-α-defensins, which each contributes three cysteines to the structure [292]. θ-defensin genes are also present in the human genome, but their expression is prevented by a premature stop codon. Their protective capacities have been shown in various infection models [293]. Synthetic θ-defensins with sequences that correspond to those encoded within the human pseudogenes inhibit the cellular entry of HIV, herpes simplex virus (HSV), and influenza A virus. The same molecules protect mice from infection by *Bacillus anthracis* spores. Rhesus θ-defensin 1 (RTD-1) was shown to protects mice from coronavirus infection.

II.3 Evolution of AMPs

The evolution of immunity involves direct interactions between the host and microorganisms. Natural selection as a response to the rapid evolution of pathogens is expected to strongly influence genes involved in these processes. Two well studied examples for immunological evolution by gene duplication and selection are the immunoglobulins and the major histocompatibility complex [294,295]. The evolution of human defensins is thought to have developed in a similar manner.

II.3.1 Evolution of human defensins

The complex evolution of defensin genes over time has lead to an exceptional cluster of paralogous genes with varying copy numbers [296,297]. In a genomics approach Schutte *et al.* used a computational search tool to identify 28 new human β-defensin genes, additional to the 4 previously described ones (DEFB1-4) [298] (Figure 23). They are organized in five syntenic loci, with the main locus on human chromosome 8p22-23, and at least 26 of them are actively transcribed. A comprehensive computational search performed by Patil *et al.* identified a cluster encoding 10 distinct human α-defensin genes and pseudogenes on chromosome 8p23 [299]. Furthermore, they performed phylogenetic analyses, which revealed two distinct clusters, implying that those might have independently evolved from two separate ancestral genes: One giving rise to enteric, and another to myeloid α-defensins. On the other hand, Xiao *et al.* suggested that all mammalian defensins are evolved from a common β-defensin-like ancestor [300]. α-defensins are thought to have originated from β-defensins by gene duplication, which probably occurred after the divergence of mammals from other vertebrate species, as they have not been identified in the latter [299]. Then, θ-defensins arose from α-defensins specific to the primate lineage. Nguyen *et al.* hypothesized that the additional diversification of α- and θ–defensins reflect the need for antiviral defense in certain mammalian and/or primate species, as it was shown that these new defensin families can prevent virus entry into host cells [301,302].

Comparative analyses of both α- and β-defensin genes found that during the divergence of primates episodes of purifying, positive and balancing selection have driven the evolution of these gene families [303-305]. Defensins are encoded in two exon genes. Comparisons among first-exon sequences, which encode a signal peptide, show little variation with an excess of synonymous substitutions. This observation is consistent with neutral evolution or weak purifying selection. In contrast, second-exon sequences, which encode the mature β-defensin peptide, show substantial divergence with an excess of non-synonymous (amino-acid-altering) substitutions. This is an indicator for the action of positive selection and implies a role for the second exon sequence in functional diversity of peptides [296,303,306]. Interestingly, the gene encoding human DEFB1 was found to be relatively similar across primates [307]. Evidence for an evolutionary mechanism of duplication followed by diversification



Figure 23: Computational identification of human β-defensin genes. Chromosomal location is indicated, except where mapping was ambiguous (A). Genes are marked as known (K), if evidence exists that they are transcribed and show antimicrobial activity; related (R), if evidence exists that they are transcribed but have not been tested for antimicrobial activity; predicted (P), if no evidence exists that they are transcribed; and pseudogenes (S), if the DNA sequence is highly similar to a β-defensin gene, but the predicted amino acid sequence lacks an ORF across the six-cysteine motif. Cysteine residues are highlighted in yellow, positively charged residues in blue and other residues in red if they are represented in greater than 50% of all predicted β-defensin proteins [298].

driven by positive selection has been discussed for α-defensin genes [308], bovine β-defensin genes [309] and amphibian genes encoding AMPs [310].

Overall, the complex patterns of defensin evolution may reflect the need to preserve their functional integrity, while at the same time favoring functional diversity to provide responses to a wide range of pathogens, as well as activities beyond their antimicrobial action.

II.3.2 Genetics of β-defensins

The genetics of defensins are of special interest because of the relationship between genetic variations with their expression level and susceptibility to disease. The β-defensin cluster shows high copy number variation (CNV), ranging from 2–12 copies per diploid genome, with 4 copies being the average in the healthy population [297]. Around 12% of the human genome is affected by CNVs [311] and the β-defensin cluster represents one of the CNV hotspots. Just like single nucleotide polymorphisms (SNPs), CNVs may affect gene expression and hence determine phenotypes and pathological conditions [312]. High copy numbers of β-defensin genes are associated with the skin disease psoriasis [313]. In the case of Crohn's disease conflicting results have been found, indicating either a predisposition to disease by high or low copy number [314,315]. Whether copy number is correlated to gene expression in the case of β-defensin is not completely clear. Hollox *et al.* found that the transcript level of DEFB2 (encoded by the DEFB4 gene) is moderately correlated with its copy number in different B-lymphoblastoid cell lines [313]. And also Fellerman *et al.* found reduced mRNA levels with lower copy numbers of the DEFB2-endocing gene in patients suffering from Crohn's disease and ulcerative colitis [314].

In addition to CNV, sequence variations (multisite variations, MSVs) represent a further level of genomic complexity. Analysis of the DEFB2-encoding gene promoter region revealed remarkably high sequence variability (~1 MSV/-41 bp) [316]. Sequence variability has to be considered in the evaluation of disease phenotypes. For example, an association study linked DEFB4 (encoded by the *DEFB104* gene) copy number and MSVs to prostate cancer, demonstrating an association of four common DEFB4-encoding gene haplotypes with disease risk in two independent patient cohorts [317].

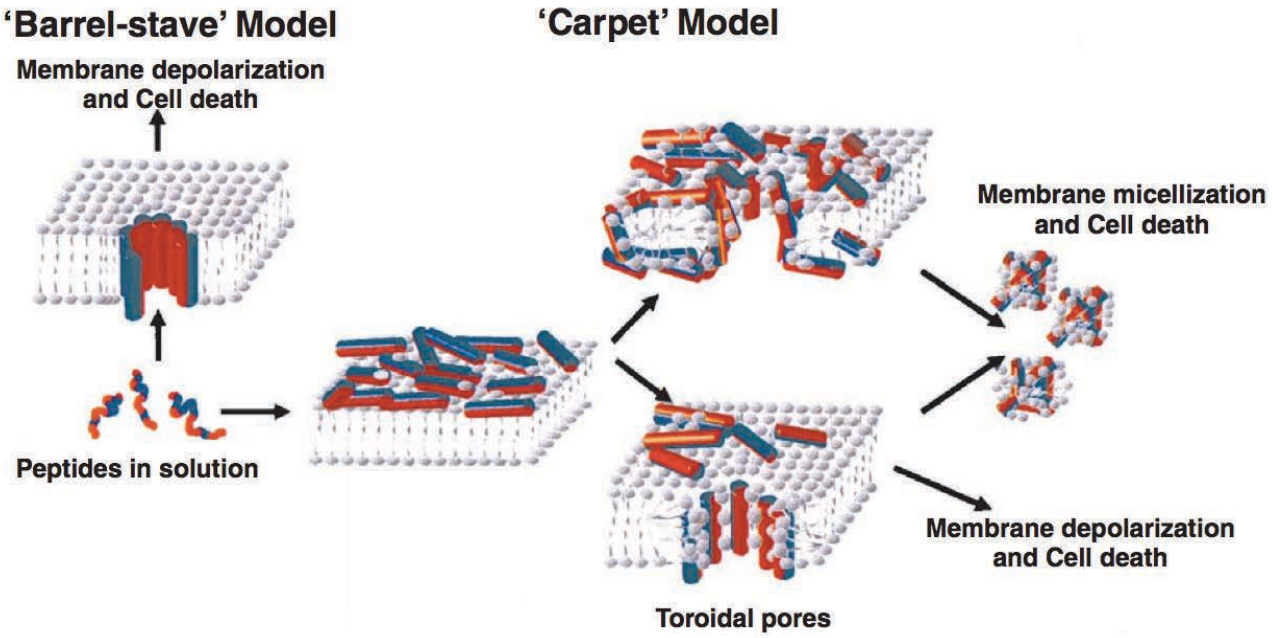


Figure 24: Mechanisms of AMPs membrane destruction. The 'barrel-stave' mechanism is characterized by the formation of transmembrane pores and depolarization followed by cell death. In the 'carpet-like' model AMPs align parallel to the outer membrane surface mainly by electrostatic interactions. After a threshold concentration of peptides has been reached, they permeate the membrane, which is followed by membrane disintegration and micellization. The 'carpet-like' model can also result in the formation of transient 'toroidal' pores, which might also lead directly to cell death (adapted from [331]).

II.4 Functions of AMPs

The more we learn about AMPs the more it becomes clear that they are more than just microbial killers. Additionally, to their own defense directed mechanisms against pathogens they recruit the second wave of effector immune cells. But at the same time as they are activating the adaptive immune response they are also controlling it via the interaction with TLRs. And when the fight is over, they are promoters of wound healing, cell proliferation and vascularization of the damaged tissues. The next few chapters will discuss the multiple important actions of AMPs in more detail.

II.4.1 Microbial killing

AMPs are destructive and inhibitory for many bacterial species and viruses. They possess a range of mechanism of bacterial lysis and prevention of virus infection, which will be discussed in this chapter. At the same time the host cells show characteristics, which protect them from their own defense arsenal.

II.4.1.1 Bacterial killing

AMPs succeed in killing many different types of Gram-positive and Gram-negative bacteria, viruses, fungi and protozoa. Some AMPs expressed by Paneth cells, such as lysozyme and PLA2s, carry out an enzymatic attack, hydrolyzing peptidoglycan or phospholipids of the cell wall [318]. Multiple techniques have been employed to establish models of the molecular mechanism of cationic AMPs, such as defensins and LL37, on their target cells [319]. The first step is always the establishment of attraction by electrostatic bonding between the AMP and structures of the bacterial cell surface. Anionic phospholipids and phosphate groups of LPS from Gram-negative and teichoic acids from Gram-positive bacteria attract cationic AMPs. The mechanism by which AMPs transverse capsule polysaccharide or cell wall structures, to reach the bacterial membranes has not really been described so far. Once they reach the outer membrane of Gram-negative or the cytoplasmic membrane of Gram-positive bacteria, they start to insert themselves into the lipid bilayer. A number of models have been proposed to explain membrane permeabilization depending on the AMP molecule and membrane structure (Figure 24). The AMP alamethicin employs a ‘barrel-stave model’, where alpha helical peptides form a barrel structure in the membrane with a central lumen [320]. Another model employed by amphipathic alpha helical AMPs in the ‘carpet model’. Peptides, such as ovispirin, accumulate in

parallel on the bilayer surface. At high concentrations they disrupt the bilayer in a detergent-like manner, eventually leading to the formation of micelles [321,322]. The 'toroidal-pore model' is employed by magainins, protegrins and melittin. Here, the polar faces of the AMP helixes associate with the polar head groups of the lipids. This induces the lipid monolayers to bend continuously through the pore so that both the inserted peptides and the lipid head groups line the water core [320,323]. All these models have the goal of inducing pores, leading to extensive membrane rupture, followed by depolarization, leakage of cellular contents, disturbance of membrane functions and finally lysis of the host cell.

Nonetheless, this is not the only killing mechanism employed by AMPs as also intracellular targets have been identified. Mechanisms have been described for peptide translocation across the membrane without causing membrane rupture [324,325]. Once inside the target cell, AMPs can disrupt important cellular mechanism such as synthesis of nucleic acids, cell wall components or proteins [326,327]. Various peptides have been shown effective in the inhibition of cell division *in E. coli*, *Salmonella* and *Shigella* species leading to the formation of elongated filaments [328-330]. The AMP mersacidin was shown to inhibit peptidoglycan biosynthesis by tempering with membrane-associated transglycosylation of peptidoglycan precursors [332]. In fungi, histatins induce the loss of ATP from actively respiring cells, disrupt the cell cycle and lead to the generation of reactive oxygen species [333]. Pyrrhocoricin and drosocin bind specifically to the heat-shock protein DnaK, thereby inhibits chaperone-assisted protein folding [334].

II.4.1.2 Anti-viral activities

AMPs possess potent antiviral activity, which they exert via different mechanism. β-defensins can suppress viral infection by disruption of the viral envelopes [268]. Additionally, they can interact with viral glycoproteins and receptors and thereby block viral binding and entry into the host cell. DEFB3 for example is an antagonist of the HIV co-receptors CXCR4 [335]. Moreover, they have been shown to interfere with cell-signaling pathways that are required for viral replication. *In vitro* studies have shown that DEFB3 can inhibit HIV replication [335].

II.4.2 Immunomodulation

Besides, their destructive capacities towards microbes, AMPs are important regulators of the innate and adaptive immune response. They can influence gene expression of cytokines and chemokines and recruit effector cells, such as neutrophils, monocytes, macrophages, immature dendritic cells and T cells, to the infection site (Figure 25). Thereby, they are able to coordinate a united response towards an infectious threat and mediate a fast resolution and reversion of harmful inflammation and tissue damage.

II.4.2.1 Chemotaxis

One way to achieve recruitment of effector cells is the induction of chemokine expression by AMPs at the infection site. LL37 for example synergistically enhances the IL1β induced production of cytokines IL6 and IL10 and macrophage chemo-attractant proteins MCP1 and MCP3 in human peripheral blood monocytes [336]. Another way is the direct interaction of AMPs with effector cell receptors to mediate their recruitment and activation. Interestingly, different AMPs seem to be specific for distinct host-target cells. Human neutrophil α-defensins are chemotactic for naïve $CD4^+CD45RA^+$ and $CD8^+$ T cells [337], immature dendritic cells, monocytes, and macrophages [338]. Human β-defensins are also chemotactic for immature dendritic cells and induce the migration of memory $CD4^+CD45RO^+$ T cells via the G protein-coupled receptor CCR6 [339]. Another study demonstrated the capacity of DEFB2 and DEFB3 to interact with CCR2, a chemokine receptor expressed on monocytes, macrophages, and neutrophils [340]. LL37 is chemotactic for neutrophils, monocytes and T cells via interaction with the G protein-coupled formyl peptide receptor-like 1 (FPRL-1) [341]. DEFB2 and LL37 are further recognized as chemoattractants for mast cells [342,343].

II.4.2.2 Modulation of TLR signaling

AMPs can regulate and balance the inflammatory responses towards microbes by influencing TLR recognition of ligands and downstream signaling. Cathelicidins have been proven especially capable of interference with TLR4 signaling. In a mouse model of allergic contact dermatitis, cathelicidin was able to inhibited TLR4-mediated

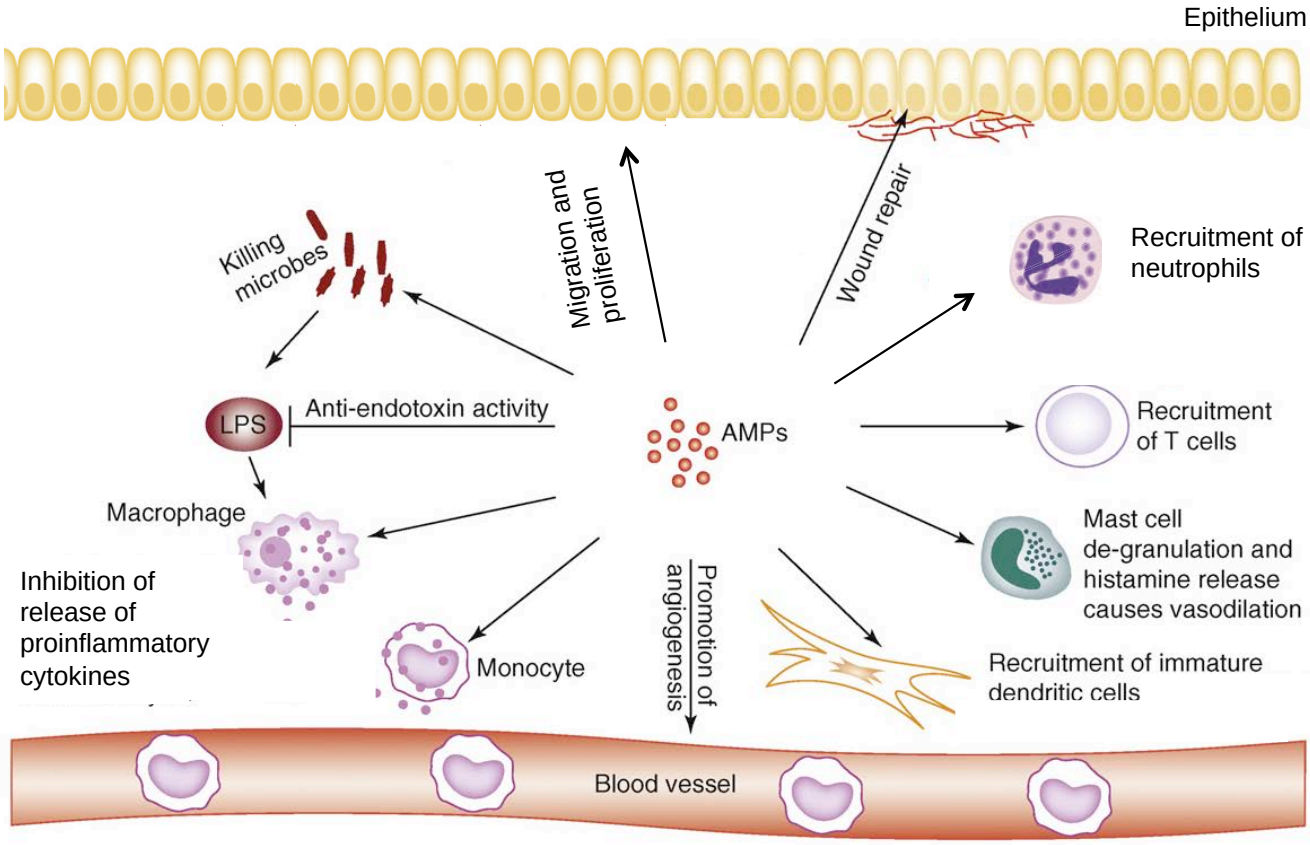


Figure 25: Immunomodulatory properties of AMPs. AMPs are able to recruit a wide range of immune cells, such as T cells, monocytes, macrophages, neutrophils and immature DCs. Furthermore, they influence the activation of TLRs and the expression of proinflammatory cytokines. Besides these mechanisms, which are involved in the resolution of an infection, they promote epithelial repair and neovasculatization (Adapted from [196]).

dendritic cell maturation and cytokine release [344]. Moreover, cathelicidin blocked the activation of TLR4 by its endogenous ligand hualuronan in murine bone-marrow derived macrophages and thereby prevented the release of macrophage inflammatory protein 2 (MIP2), which is chemotactic for polymorphonuclear leukocytes [345]. In humans, LL37 has been found to selectively suppress TNFα release and expression of proinflammatory genes upregulated by NF-κB in monocytes and macrophages stimulated by LPS [346]. It has been proposed that cathelicidin exerts the inhibition of receptors such as TLR4 by modifying the membrane microdomain containing the receptor, thereby changing the receptor function and ligand response. Furthermore, LL37, among other AMPs, can act as a scavenger receptor for LPS, thereby preventing its binding to TLR4 [347,348]. This mechanism of LPS-neutralization conveys protection against septic shock in mice [349].

II.4.3 Promotion of wound healing

AMPs are not only at the front of immune defenses against invading pathogens, but they are also involved in cleaning up the battlefield and taking care of the casualties. Beta defensins and LL37 readily accumulate at wounds and their role in vascularization and wound healing is widely established [350351]. DEFB2 was shown to stimulates chemotaxis, proliferation and tube formation of human endothelial cells and speed-up wound closure [351]. LL37 induces keratinocyte migration and re-epithelialization of skin wounds via transactivation of the epithelial growth factor receptor (EGFR) transactivation [352]. Furthermore, LL37 promotes angiogenesis and neovascularization by binding to FPRL-1 expressed on endothelial cells [353]. Additionally, LL37 shows anti-fibrotic effects by inhibiting collagen expression in dermal fibroblasts, thereby promoting normal wound repair [354].

II.4.4 Why are host cell membranes protected?

The membranes of bacteria and the eukaryotic host differ in important characteristics, which convey protection from AMPs attack to the host cell [195,355] (Figure 26). First of all, the outer leaflet of mammalian cell membranes is exclusively composed of electrically neutral, zwitterionic phospholipids, mainly phosphatidylcholine and sphingomyelin. Negative charges are located to the inner leaflet of the membrane and not exposed at the surface. Second the eukaryotic membrane contains stabilizing cholesterol, which has been shown to reduce the

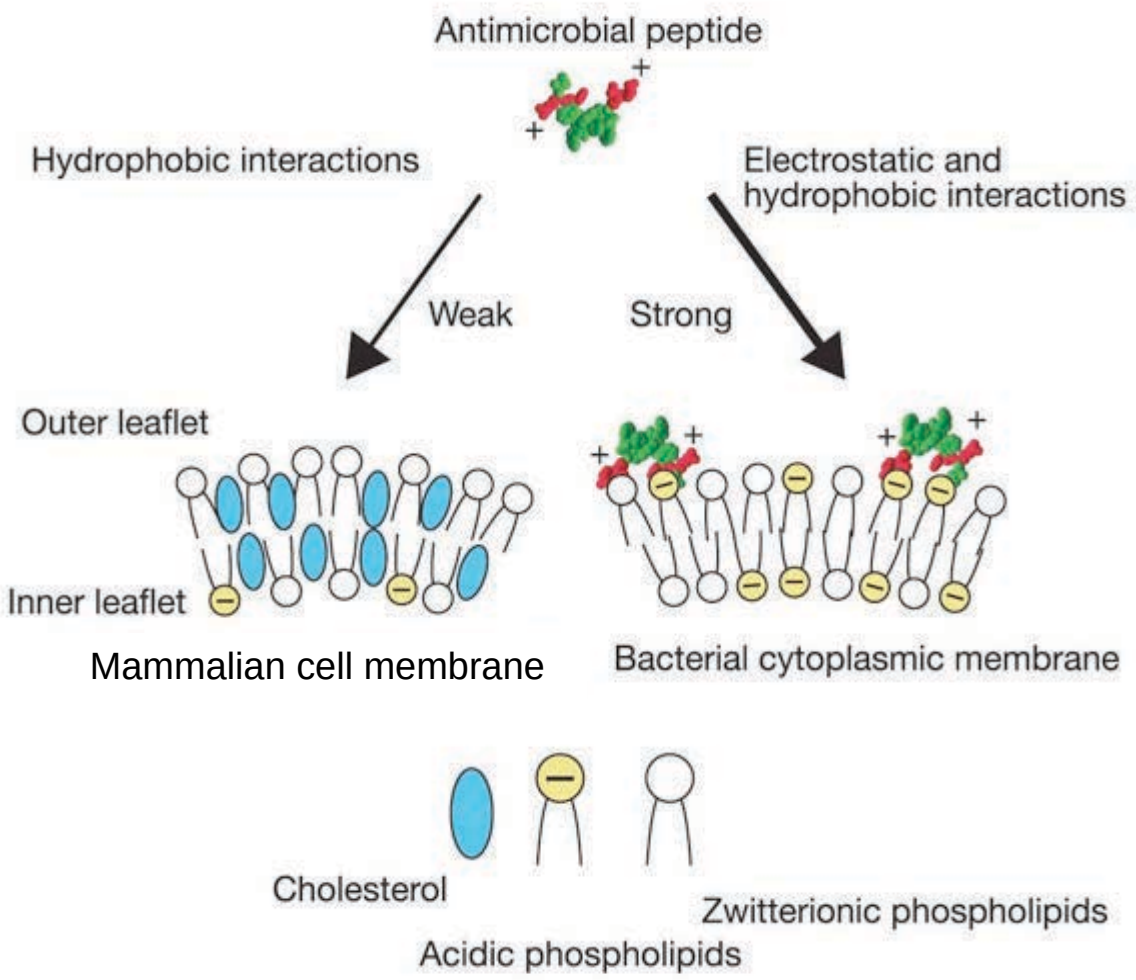


Figure 26: Differences between bacterial and mammalian membranes protect host cells from AMPs attack. Cationic AMPs preferentially bind to bacterial membranes with abundant acidic phospholipids carrying a negative charge. The outer leaflets of mammalian cell membranes are exclusively composed of zwitterionic phospholipids with neutral charge. The presence of cholesterol additionally protects mammalian membranes against AMPs (adapted from [195]).

activity of AMPs towards the lipid layer. Moreover, it was shown that the activity of AMPs is blunted by physiological salt concentration (e.g. 150 mM NaCl), increased presence of divalent cations and serum proteins [356]. This might prevent destruction of host cells in a physiological environment but still allow killing of bacteria, which change their membrane structures and gene expression profile in adaptation to the host environment [357].

The same properties, that protect healthy host cells, are aberrant in some cancer cells and thereby make them targets for the destruction by AMPs. For example, the membrane net negative charge is increased in malignant cells by the accumulation of anionic molecules, such as phosphatidylserine, O-glycosylated mucins, sialylated gangliosides and heparin sulfate [358]. Especially during cell transformation phosphatidylserine molecules will present themselves on the outer membrane leaflet, counteracting the typical phospholipid asymmetry of the membrane. Moreover, most cancer cells show increased membrane fluidity, making them more susceptible for destabilization by AMPs [359]. It has also been suggested that the enlarged cell surface area of malignant cells, formed by elevated number and distorted features of microvilli, confers a higher contact possibility with AMPs molecules [360]. Additionally, lysis of mitochondrial membranes have been described to lead to the release of cytochrome c and eventually apoptosis [361]. Many natural and synthetic AMPs are being tested for cancer treatment [362]. However, not all AMPs are active against cancer cells and we are just beginning to understand the factors that allow certain AMPs to recognize and lyse neoplastic cells. Understanding efficacy and selectivity of those peptides, as well as the identification of specific targets that are expressed and presented within a certain tumor type will be crucial in the process of drug design in the future.

II.5 Bacterial resistance to antimicrobial peptides

In order to survive, bacteria have evolved under selective pressure to stay on top of their host's defense mechanisms. The same is true for developing resistance to AMPs. At the same time this presents an enormous challenge, as the targets of AMPs are essential to bacterial integrity and survival, such as the bacterial

membrane. Bacteria differ in their intrinsic susceptibility to AMPs and pathogenic species have come up with their own remarkable protection strategies. Recent evidence indicates that bacterial resistance to AMPs is under control of global regulation systems, which coordinate expression of virulence factor. Those bacterial two-component signal transduction systems usually consist of a membrane-bound histidine kinase, which senses a specific environmental stimulus, and a response regulator, which mediates the cellular response [363,364]. These systems enable bacteria to sense, respond, and adapt to a wide range of environmental cues encountered in different tissue niches of their host. One example for such systems is the PhoP/PhoQ two-component regulatory system of *Salmonella enterica*, which is involved in AMP resistance [365]. These mechanisms will be discussed in greater detail in the chapter below.

Moreover, mutagenesis approaches were used to target genes of major AMP resistance mechanisms to show that loss of the resistance phenotype translates to decreased virulence *in vivo.* Direct comparisons of resistant wild-type bacteria with AMP-sensitive mutants in *in vivo* infection models provided validation of the contribution of resistance mechanism to pathogenesis and shed light on the involvement in human disease conditions [366].

II.5.1 Secretion of AMPs degrading enzymes

Destruction of AMPs can be achieved by secretion of degrading enzymes, as demonstrated for species belonging to *Staphylococci* (Figure 27 D). *S. aureus* metalloproteinase (aureolysin) and glutamylendopeptidase (V8 protease) are capable of cleavage and thereby inactivation of LL37 in a time- and concentration-dependent manner [367]. The skin commensal and leading nosocomial pathogen *Staphylococcus epidermidis* efficiently inactivates the anionic AMP dermcidin through its metalloprotease sepA [368]. LL37 was also shown to be degraded by bacterial supernatants containing GAS cysteine proteinase, *Proteus mirabilis* metalloprotease, or *Enterococcus faecalis* gelatinase [369]. Even more elegant is the mechanism employed by *P. aeruginosa.* The pathogen stimulates the accumulation of host cysteine protease cathepsins B, L and S in the airway fluid, which mediate degradation and inactivation of DEFB2 and DEFB3 [370].

II.5.2 Modifications of the bacterial membrane

The negatively charged surface of bacteria presents a welcoming site for cationic AMPs attack (Figure 26). So, one important protection mechanism employed by both Gram-positive and Gram-negative bacteria is the modification of anionic surface constituents with cationic molecules in order to repel cationic AMPs.

LPS covered Gram-negative bacteria prevent AMPs binding by the synthesis of LPS with specific lipid A modifications (Figure 27 B). *Salmonella* spp. can produce hepta-acetylated lipid A via the addition of palmitate by the bacterial factor PagP. Moreover, it can add phosphate and phosphoethanolamine to the core polysaccharide and modify lipid A phosphate groups with ethanolamine and 4-aminoarabinose [371]. These changes are implemented via the two-component signal transduction systems PhoP-PhoQ and PmrA-PmrB. Interestingly, both these systems were found to be upregulated upon the presence of subinhibitory concentrations of AMPs [372]. A PmrAB-like signal transduction system and Ara4N modification of lipid A has also been identified in *P. aeruginosa,* a common airway pathogen in cystic fibrosis patients [374]. Additionally, *P. aeruginosa* synthesizes a hexa-acetylated lipid A with palmitate and aminoarabinose modifications [375].

Gram-positive bacteria neutralize their surface charge by modifying teichoic acids in their cell wall with D-alanyl groups or by including positively charged phospholipids in the cellular membrane (Figure 27 A). *Staphylococci* species employ the three-component antimicrobial peptide-sensing system to regulate synthesis of amino acids, such as L-lysine for the decoration of membrane lipid phosphatidylglycerol or D-alanine for the decoration of teichoic acid [376,377]. D-alanylation is achieved by gene products of the *dlt* operon, which was also identified in other pathogens, such as Group B *Streptococcus* (GBS) [378], *Bacillus subtilis* [379] and *L. monocytogenes* [380], where it exerts the same role in AMPs resistance.

The human gastric pathogen *H. pylori* modifies host cholesterol by glycosylation and incorporates it onto its surface. Cholesterol-grown *H. pylori* cells showed an over 100-fold increased resistance to eight antibiotics and LL37 [381].

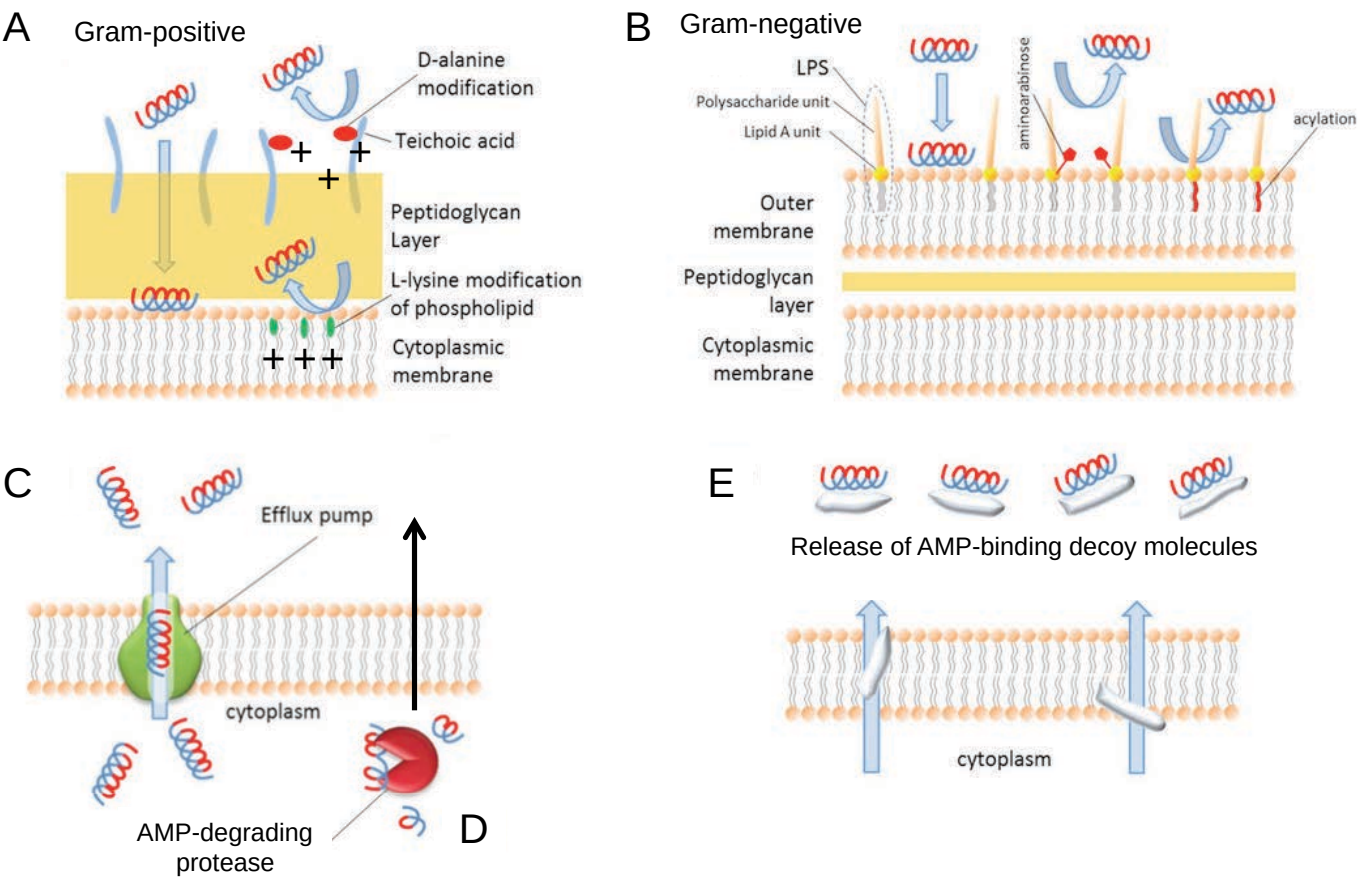


Figure 27: AMP-resistance mechanism of bacteria. Bacteria have evolved several mechanisms to resist destruction by AMPs. Gram-positive bacteria for example modify their surface teichoic acid and membrane phospholipids with positive charges, to repel cationic AMPs (A). Gram-negative bacteria modify their LPS molecules with aminoarabinose or acylation of lipid (B). Furthermore, already intracellular AMPs can be extruded by efflux pumps (C) or degraded by proteases, which can also be secreted by some species (D). Some bacteria release decoy proteins to bind and block AMPs (E) (adapted from [373]).

II.5.3 Trapping of AMPs

Pathogens have developed different mechanism to bind and thereby neutralize AMPs (Figure 27 E). *S. aureus* for example secretes an AMP-binding factor called staphylokinase. This protein was shown to directly bind HNP1 from neutrophils and inhibit its bactericidal effects towards *S. aureus* [382]. GAS strains expressing the surface protein M1 exhibit increase level of resistance to LL37 as compared to strains expressing other M proteins. Studies showed the AMP-binding capacity of the hyper variable C-terminus of M1 protein [383]. Moreover, GAS secrete an additional protein called SIC, which can bind and inactivate LL37 and DEFB1 [384].

Another astonishing mechanism is the exploitation of negatively charged proteoglycan molecules from host epithelial cells, which work as a decoy to eliminate AMPs. Several pathogens, including GAS, *P. aeruginosa* and *E. faecalis,* have been shown to release proteases that degrade host cell-surface proteoglycans and release dermatan sulfate, which binds human α-defensins [385].

II.5.4 Active efflux of AMPs

Energy-driven drug efflux systems have already been accounted for resistance to antibiotics (Figure 27 C). Now some of these systems are discovered to also expel AMPs. Examples are the MtrCDE system of *Neisseria gonorrhoeae* [386], systems encoded by the *sap* locus in *Salmonella enterica* [387] and a homologous locus in *Haemophilus influenza* [388], and the QacA efflux pump in *S. aureus* [389].

II.5.5 Downregulation of AMPs expression

Specific inhibition of AMP expression by virulence factors might be the best strategy to obtain resistance to destruction by AMPs. *H. pylori* is able to temper with the expression of gastric DEFB3, which is rapidly induced during early infection and highly active against *H. pylori in vitro*. However, during prolonged infection, DEFB3 was found subsequently downregulated, in dependence of the virulence factor CagA [390].

Some *Shigella* species are able to shut down expression of the antibacterial peptides LL37 and DEFB1, as observed in biopsies from patients with bacillary dysenteries and in *in vitro* infections *of* epithelial and monocyte cells. This seems to be mediated via the *Shigella* virulence plasmid [391]. Another study in the human

intestinal xenograft model could show that virulent *S. flexneri* suppress transcription of DEFB3 in dependence of the virulence regulator MxiE [392].

II.5.6 Why are AMPs still successful?

After thousands of years of coevolution with pathogens, AMPs still confer successful broad-spectrum protection. Several important points have to be mentioned in the discussion of AMP superiority to bacterial resistance. First of all, the development and expression of resistance genes could involve biochemical modifications that are metabolically too costly for maintenance. Especially considering the main targets of cationic AMPs, the bacterial membrane and cell wall. The functional and structural integrity of these structures are vital and therefore alterations do not allow changes in membrane composition or lipid organization, which could provide protection against AMPs [195,196]. This might be the reason why many species have developed inducible resistance systems that don't have to be activated all the time.

Second, AMPs possess a relative intrinsic resistance to proteolytic cleavage by bacterial enzymes. Proline-rich sequences, for example in cathelicidins, confer resistance to serine proteases [393]. Additionally, most AMPs possess a nondescript sequence of amino acids and lack unique epitopes for specific bacterial protease recognition. This implies difficulties to design a destructive enzyme, which would not also target host proteins needed for bacterial attachment or bacterial proteins. Additionally, the collective repertoire of different AMPs lanced by the host presents a structural diverse cocktail of death. Even though some bacteria express AMP-directed proteases for the elimination of one sort of peptide this does not present the solution for survival in this case. This feature represents an important advantage towards classical administered antibiotics, which are often expected to act on the basis of a single compound, which can easily be overcome by bacteria. Besides, the complexity, expression of AMPs at an infection site leads to extremely high local concentrations that are difficult to overcome.

II.6 Relationship between AMPs and human pathologies

As AMPs are at the front line of innate immunity, their expression is vital for protection against infectious threats and control of homeostasis. Therefore,

deregulation of their expression can lead to serious deficiencies in fighting microbial invasion and controlling inflammatory processes. To date, multiple pathologies involving decreased, but also increased AMP expression have been described [394] (Table 2). Besides, their importance in protection against deadly pathogens, such as *Shigella*, *Salmonella* and HIV, their role in the control of commensal, which present a constant threat of opportunistic infections, is widely recognized [395]. Polymorphisms, variations in gene copy numbers, but also deregulation and manipulation by pathogens have been described in association with susceptibility and resistance to infection.

II.6.1 Inflammatory bowel diseases - Crohn's disease and ulcerative colitis

In the scope of work presented in this thesis the role of AMPs in gut homeostasis and protection is especially highlighted. An intensively studied disease condition of our time in the gut is inflammatory bowel disease (IBD) [396,397]. This disease includes two chronic inflammatory conditions: Crohn's disease (CD) and ulcerative colitis (UC) (Figure 28). Both are characterized by a severe and recurrent tissue inflammation, leading to frequent diarrhea, ulcerations and fistula formation. The main difference between the two pathological conditions is their location and degree of inflammation. CD can manifest itself in any part of the gastrointestinal tract, while UC is restricted to the colon and the rectum. Inflammatory processes in CD are transmural, while inflammation in UC is superficial and restricted to the mucosa. IBD patients suffer from grave limitations in quality of life and treatment is based on anti-inflammatory drugs and sometimes antibiotics. No clear etiology has been identified so far and the complexity of the disease is just beginning to be unraveled. Various predisposing genetic host factors have been discovered [398], but environmental factors such as recurrent intestinal infections and smoking [399] as well play a crucial role in development of the disease. The increased incidence of CD over the last 50 years has also inspired a link with the hygiene hypothesis. In this regard the reduced contact with microbes during the development of the immune system is discussed as an explanation for an inappropriate response to gastrointestinal infection later in life [400].

The major process underlying IBD is a loss of tolerance to commensal microbes and disturbance of the intestinal homeostasis. Alterations in the

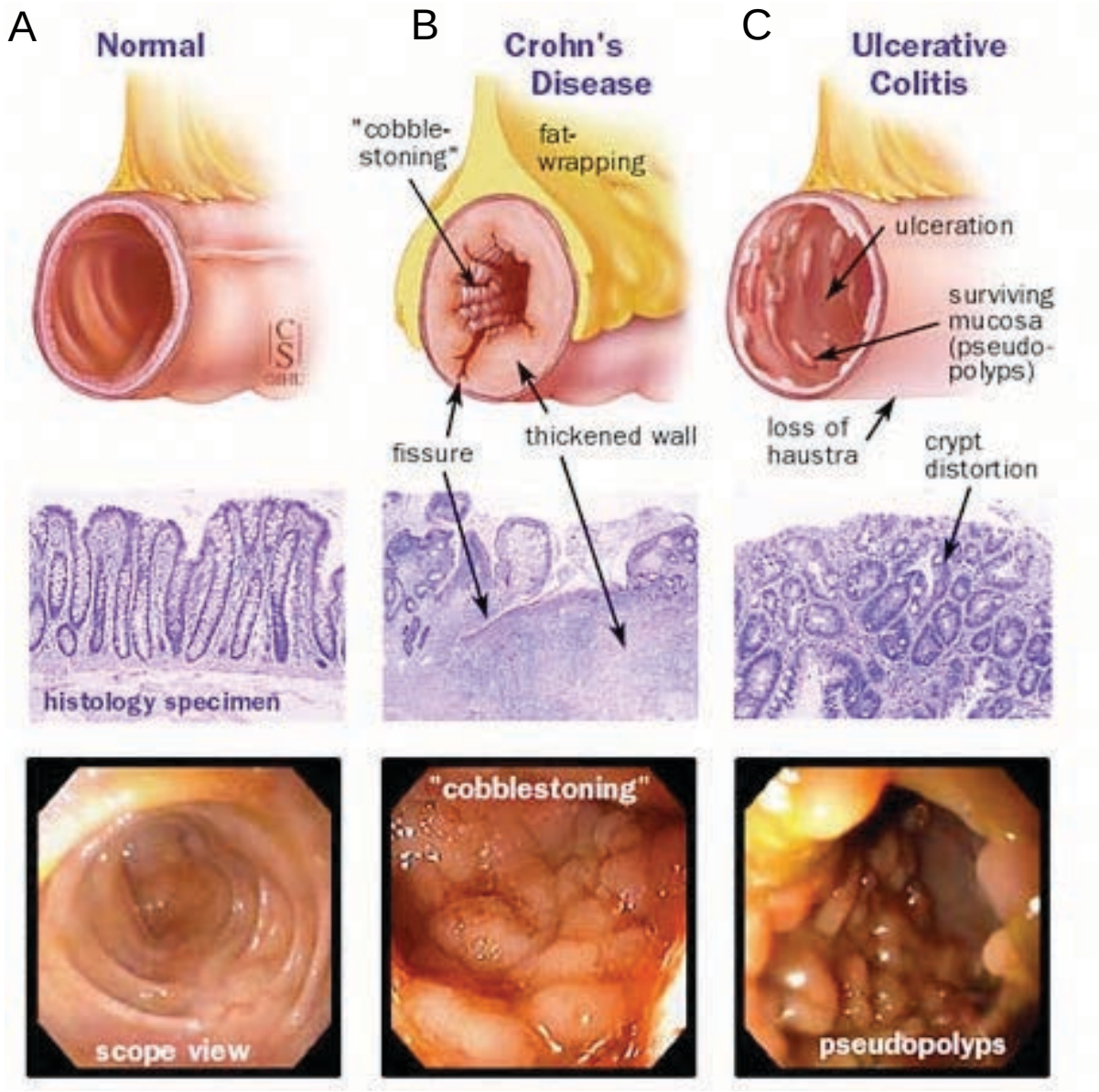


Figure 28: Pathological characteristics of inflammatory bowel diseases. These schematics and pictures compare the appearance of the colon (top panes), the histology (middle panel) and endoscopic views (bottom panel) of normal (A), Crohn's disease (B), and ulcerative colitis (C) patients. Crohn's disease colon is characterized by appearance of so-called "cobblestoning" of the mucosa, thickening and fissures of the wall and fat wrapping of the intestine (B). UC colon is characterized by the appearance of pseudopolyps, ulcerations and crypt distortion (C). (Adapted from Johns Hopkins Medicine, Gastroenterology & hepatology)

composition of the gut microbiota may allow an overgrowth of harmful species, which are capable of causing inflammation. At the same time failure of the host immune responses may allow for bacterial translocation and inflammatory processes. The question of what comes first in this scenario is still unsolved, highly debated and the answer could be as difficult as solving the question about the egg and the hen. As defensins are the major defender of the epithelium in the intestine their role in IBD development is under vigorous investigation. Important differences in their local expression and inducibility have been identified in CD and UC, which might explain a different disease etiology. Present data suggest that while in UC β-defensins are upregulated accordingly to an inflammatory stimulus, their expression is deregulated in CD, which likely contributes to the disease development.

II.6.1.1 The role of α-defensins in IBD

Interestingly, colonic epithelial cells from patients with UC displayed a significant increase of HD5 and HD6 mRNA levels [402]. The expression of α-defensins in the colon might be surprising, but immunohistochemistry experiments identified metaplastic Paneth cells, which can arise due to chronic intestinal inflammation in UC [403,404]. In contrast, case-control studies of ileal CD found reduced levels of HD5 and HD6 in patients [405,406]. This reduction can be linked to mutations in multiple factors involved in the regulation of their expression. One way to activate HD5 and HD6 expression in Paneth cells is via pattern recognition receptors. Genetic studies suggest a role for the Nucleotide-binding oligomerization domain-containing protein 2 (NOD2) pattern recognition receptor as a major susceptibility factor in a subgroup of CD patients [407,408]. NOD2 CD-associated genetic variants lack intrinsic recognition of its ligand muramyl dipeptide (MDP), a conserved structure in bacterial peptidoglycan [409] (Figure 29). The relationship between NOD2 genotype and α-defensin expression in Crohn's disease patients is under heavy debate. While two studies linked significantly lower levels of HD5 and HD6 mRNA in ileal CD patients with NOD2 mutations [410,411] a different study claimed that there is no significant association between α-defensin expression and NOD2 genotype [412]. Simms *et al.* explain the reduction in defensin expression as a result of loss of Paneth cells due to tissue damage. Though these results seem contradicting they don't have to be mutually exclusive. A combination of genetic predisposition and tissue destruction by

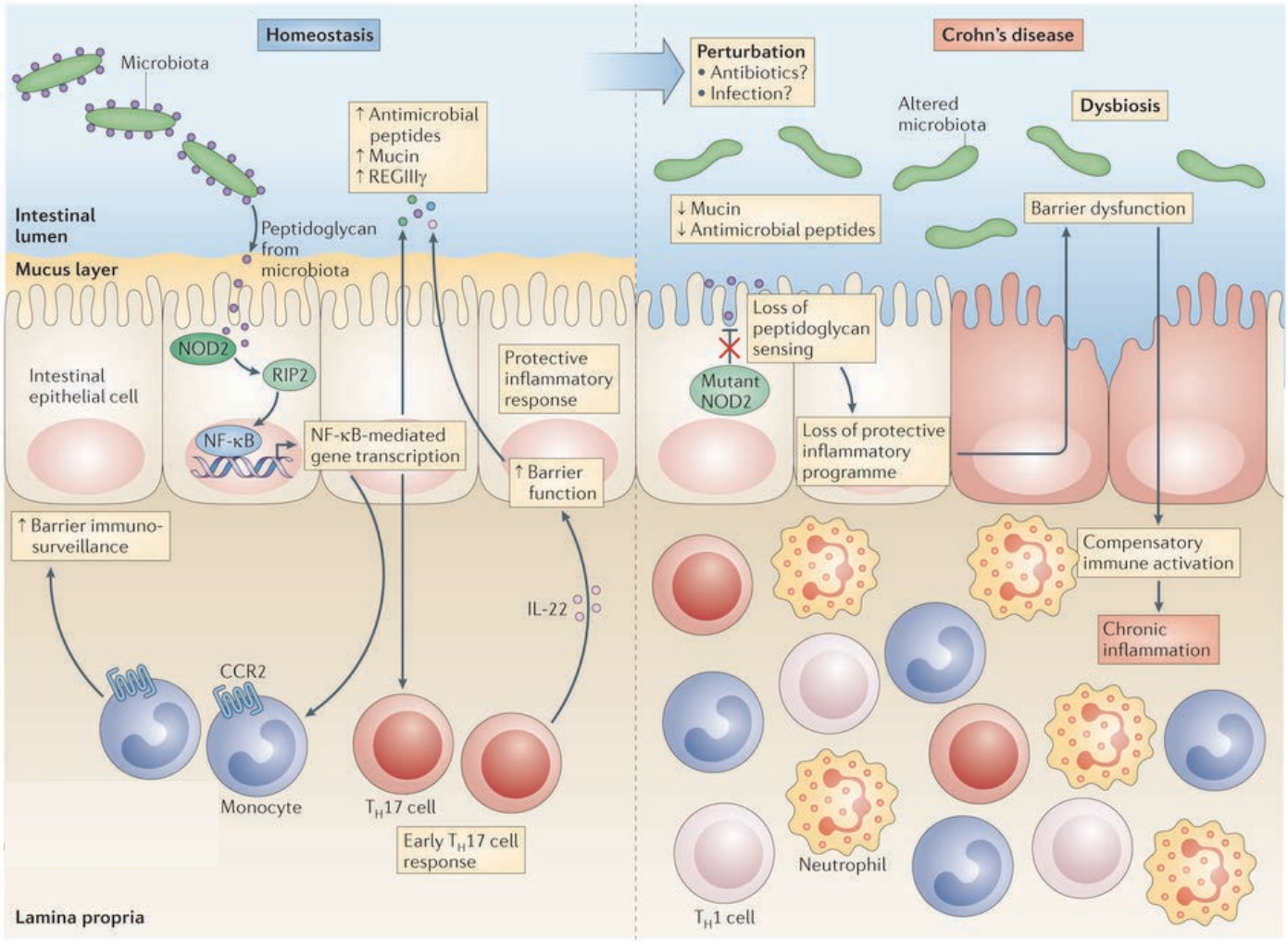


Figure 29: The relationship between NOD2, AMPs and Crohn's disease. NOD2 plays a major role in the maintenance of intestinal homeostasis, as it detects bacteria derived peptidoglycan and activates a physiological inflammatory program via activation of kinase receptor-interacting protein 2 (RIP2) and NF-κB (left panel). This leads to the production of antimicrobial peptides and mucins, which enforce the gut barrier. In this scenario, an early Th17 cell response enhances barrier protection by inducing the production of IL22 and the REG3γ. Monocytes recruited by CCL2 take up the role of barrier surveillance. In Crohn's disease mutations in the NOD2 gene, together with homeostasis perturbations, such antibiotics or infection, have been described as causes for disease (right panel). In this scenario, the protective inflammatory program is disturbed, the barrier is weakened by reduced mucins and AMPs production and eventually the microbiota composition is altered. Additionally, compensatory immune activation through other pathways then drives chronic inflammation and leads to the development of severe disease. (Adapted from [401])

inflammation can be imagined. In mice, NOD2 was shown to be responsible for the expression of intestinal cryptdins. This is underlined by the fact that NOD2-deficient mice exhibit susceptible to bacterial infection via the oral route but not through intravenous or intraperitoneal challenge [413].

Furthermore, mutations of proteins involved in Paneth cell function and secretory capacities have been identified in CD patients. Cadwell *et al.* discovered a risk variant of the autophagy related 16 like 1 (ATG16L1) protein, which was accompanied by Paneth cells abnormalities in patients [414]. This protein has essential functions in granule exocytosis and therefore the peptide export from secretory cells. Impaired AMPs secretion by Paneth cells was also linked to the X-box binding protein 1 (XBP1) transcription factor [415]. Deletion of XBP1 in a mouse model resulted in Paneth cell dysfunction and spontaneous enteritis. Another genetic associate to Paneth cell secretory capacity and CD has been made with the intermediate conductance calcium-activated potassium channel protein (KCNN4) [416]. Mutations in the wingless-type (Wnt) signaling pathway, which regulates epithelial proliferation and Paneth cell maturation are also suspected to play a role in CD etiology [417,418].

II.6.1.2 The role of β-defensins in IBD

Not only α-defensins, but also β-defensins play a crucial part in IBD development. Results for the constitutively expressed DEFB1 in this regard are contradicting. Two patient studies found no difference in DEFB1 expression in the colon in UC [402,419], while another patient study by Wehkamp and colleagues showed significantly decreased DEFB1 expression in both CD and UC patients [420]. As DEFB2 is inducible by several inflammatory stimuli, it is not surprising that mRNA levels were increased in patients suffering from IBDs compared to healthy controls. Expression was especially high in inflamed areas of the tissue, with a significantly greater upregulation in UC compared to CD [402,420]. This goes hand in hand with a reduced antimicrobial activity in colonic CD compared to UC [421]. An *in vitro* study in primary keratinocytes showed that NOD2 also mediates the induction of DEFB2 by its ligand MDP via transcription factors NF-κB and AP1. And overexpression of the most frequent NOD2 variant associated with Crohn's disease in human embryonic kidney 293 cells resulted in defective induction of DEFB2 by MDP [422]. So far the relationship between NOD2 mutations and DEFB2 has not been confirmed in CD patients. A

study performed by Fellermann and colleagues also linked decreased copy number of the DEFB2 gene (<4) to a significantly lower DEFB2 expression and increased risk for the development of colonic CD [314].

II.6.1.3 The role of LL37 in IBD

Only few studies so far have investigated the role of LL37 in the development of IBDs. Schauber *et al.* reported an increase in cathelicidin expression in inflamed and non-inflamed mucosa in patients suffering from UC but not in CD [423].

II.6.2 The role of AMPs in enteric infections

AMPs are at the front line of the intestinal immune response. Therefore it is not surprising that some common enteric pathogens have developed strategies to dampen AMPs expression. The ability of *S. flexneri* to turn off LL37, DEFB1 and DEFB3 expression was shown in human polarized intestinal epithelial cells and in an *in vivo* model of human intestinal xenotransplants in mice [392]. *S.* typhimurium downregulates expression of cryptdins and lysozyme in mice [424]. On the contrary, overexpression of human HD5 in transgenic mice protects them against oral challenge with *S.* typhimurium [425]. These examples underline the importance of intestinal AMPs in the defense of the gut epithelium and therefore the host's integrity.

II.6.3 The role of AMPs in diseases concerning the lung

The epithelial surface of the lung comprises 30-50 m^2, which also directly face the environment via the connection with the nasal and oral cavity and the incoming airflow [426]. Therefore it also faces bacterial colonization and challenge. The discovery and description of the lung microbiome is relatively recent and just beginning to be unraveled [427]. The bacterial numbers are much lower than in the gut microbiome, but culture-independent studies found that lungs of healthy never-smokers are inhabited by communities composed of diverse types of bacteria, viruses and fungi [428]. In order to protect the host from invasion of microorganisms, lung epithelial cells and local inflammatory cells produce AMPs such as defensins and cathelicidin [429]. The understanding of the lung microbiome, together with the defense mechanisms, such as AMPs, could help do decipher the disease states of the lung, which are often associated with an infectious component.

II.6.3.1 Cystic fibrosis

Patients suffering from cystic fibrosis, an autosomal recessive genetic disease, possess increased AMP levels in their respiratory tract secretions. Nonetheless, the activity of AMPs is strongly inhibited by the unnaturally high salt concentrations present at the apical side of the epithelium in the disease condition [430,431]. This is the result of the defective cystic fibrosis transmembrane conductance regulator (CFTR), which regulates the transport of chloride and sodium ions across epithelial membranes [432]. Dampened AMPs activity allows for bacterial colonization and recurrent pneumonia with low virulence organisms, such as *P. aeruginosa,* eventually leading to death from lung damages. Lowering of the salt concentration of cystic fibrosis airway fluid restored its killing activity, underlining the importance of AMPs in fighting airway infections [430].

II.6.3.2 Asthma and chronic obstructive pulmonary disease (COPD)

DEFB1 is constitutively expressed in the airway epithelium, and is upregulated in response to infection. Several research teams have identified a role for polymorphisms in the DEFB1 gene in obstructive airway diseases with chronic inflammation of the respiratory tract. Asthma for example is characterized by reversible airflow obstruction, bronchial hyperresponsiveness and bronchospasms. Airway inflammation was shown to be involved in the etiology, pathogenesis, and clinical course of the disease [433]. Several case-control studies in cohorts of different ethnicities have associated SNPs in the DEFB1 gene with the pathogenesis of asthma [434]. Similar observations were made in cohorts concerning COPD [435,436], a lung disease characterized by poor airflow, sputum production and inflammatory processes, which has prior been related to cigarette smoke or other irritants, but also to childhood respiratory tract infections [437,438].

II.6.3.3 Tuberculosis

Several studies by Rivas-Santiago and colleagues showed the involvement of AMPs in the battle of lung tuberculosis. They found an increase in DEFB2 and LL37 in alveolar macrophages, monocytes, neutrophils and epithelial cells when infected with *Mycobacterium tuberculosis in vitro* [439,440]. Lui and colleagues further investigated the mechanism of LL37-conferred protection against *M. tuberculosis*. They found a

Condition	Changes in AMP[a]
Atopic dermatitis	↓ LL-37, HBD-2, and dermcidin
Atopic eczema	↓ HBD-2, HBD-3, and LL-37
Thermal injury	Lack of HBD-2 production
Rosacea	↑ LL-37 peptide form
Psoriasis	↑ LL-37
Infectious diarrhea	↓ α-defensin
Crohn's disease	↓ HBD-2 mRNA
Diabetes type I	SNPs in HBD-1; ↓ LL-37
Oral bacterial infection in morbus Kostmann	↓ LL-37, ↓ human neutrophil peptides
Chediak-Higashi syndrome	↓ AMP in neutrophil granules
HIV-1 infection risk	DEFB1 polymorphism
Tuberculosis	↓ mBD-3 and mBD-4; progressive disease ↑ mBD-3 and HBD-2; latent infection ↑ LL-37 and *CAMP*; progressive disease

[a] ↓, diminished production; ↑, augmented production.

Table 2: Deregulation of AMP expression in human pathologies. Multiple and various diseases have been associated with a diminished or augmented production of AMPs. Additionally, polymorphisms have been identified for certain AMPs in pathological conditions. (Adapted from [395])

TLR2/1-dependend upregulation of vitamin D receptor and the vitamin D-1–hydroxylase genes in human macrophages, leading to induction of the antimicrobial peptide cathelicidin and killing of intracellular bacteria. This protective mechanism was completely abrogated by the use of siRNA against cathelicidin, which lead to enhanced intracellular growth of mycobacteria [441]. Their results were undermined by a population study in African-American individuals, which are known to have increased susceptibility to *M. tuberculosis*. Serological tests showed low 25-hydroxyvitamin D levels, which were inefficient to induce cathelicidin messenger RNA production [442].

II.6.4 The role of AMPs in disease of the skin

The 2 m^2 of skin covering our most exposed body surface are heavily colonized by microbes [443] .The skin environment is naturally acidic with a pH of 4-4.5 due to lactic acid in our sweat and produced by skin microbiota [444]. This environment guarantees a very specific bacterial flora and on the other hand prevents colonization with many unwelcome guests. Additionally, AMPs such as dermcidin, psoriasin and LL37 are the main defenders of our skin. The direct exposure to the sun promotes the vitamin D mediated boosting of LL37 expression [445]. But AMPs in the skin do not only have protective effects; their dysregulation can lead to severe disease and even autoimmunity.

II.6.4.1 Atopic dermatitis

Keratinocytes express cathelicidin and β-defensins at low levels in non-inflamed skin, but their expression is rapidly increased upon injury or inflammation. Additionally, the AMP dermcidin 1 is constitutively produced in sweat glands. Atopic dermatitis (AD) and atopic eczema are two skin diseases, which are characterized by recurrent infections and cutaneous inflammation. AMPs induction is reduced in patients suffering from these pathologies, leaving them vulnerable to microbial colonization [446-449]. A mechanism for transcriptional control of LL37 expression in AD has been proposed, based on the overexpression of Th2 cytokines IL4 and IL13 in AD skin [450]. A study by Howell *et al.* reported inhibition of TLR3-induced LL37 expression by IL4 and IL13 mediated signaling through signal transducer and activator of transcription 6 (STAT6) [451]. In turn, STAT6 induces the expression of suppressor of cytokine signaling 1 (SOCS1) and SOCS3, which inhibit NF-κB binding to consensus target

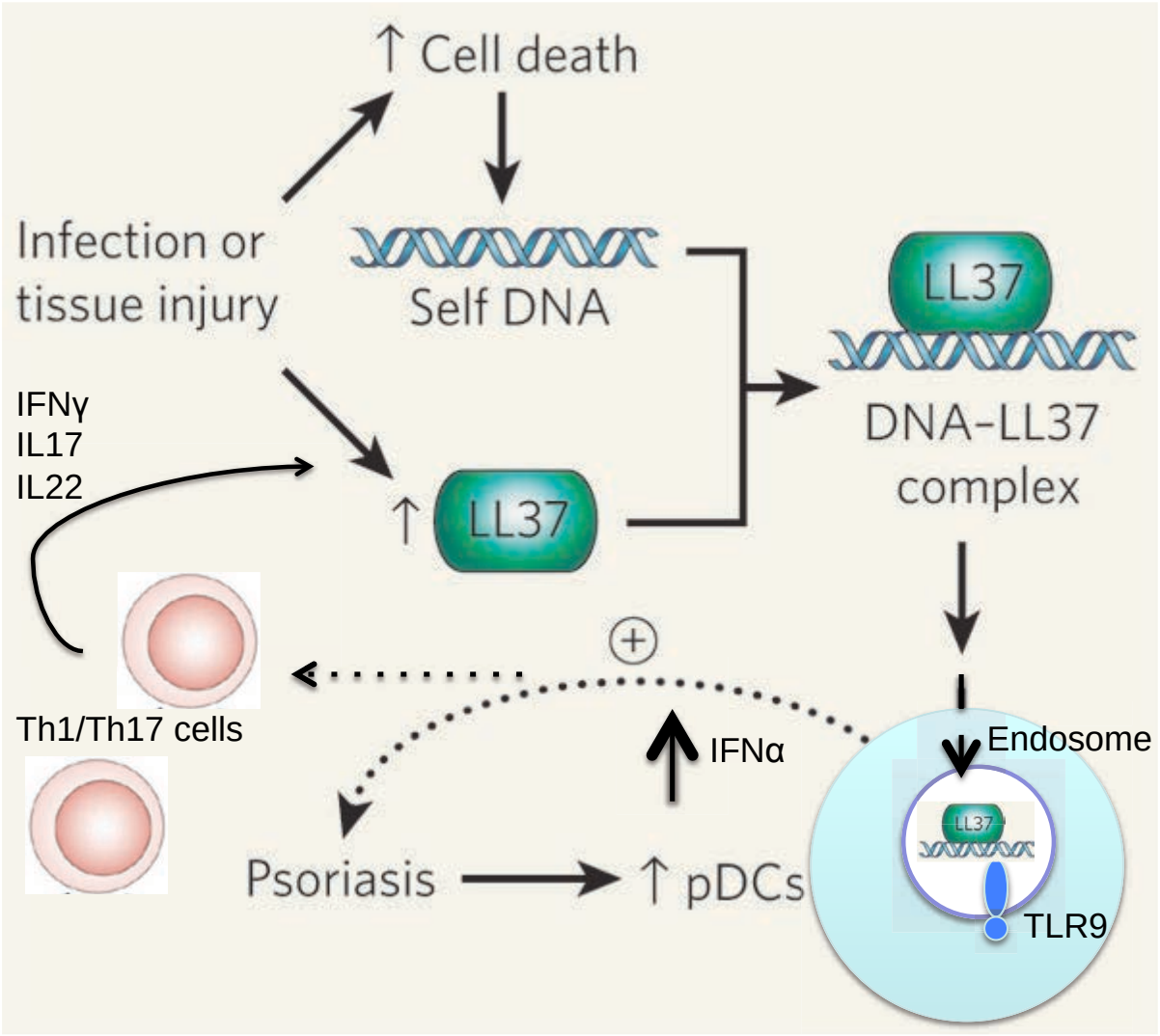


Figure 30: The inflammatory cycle of psoriasis. In response to microbial infection or tissue injury, LL37 is produced locally in the skin. In the inflammatory skin condition of psioriasis LL37 forms complexes with self-DNA, which is released from dying cells. In normal skin, these DNA–LL37 complexes probably remain undetected and inconsequential. But in the presence of plasmacytoid dendritic cells (pDCs), which accumulate in the skin lesions of patients with psoriasis, these complexes are recognized by TLR9 in endosomes and trigger a strong IFN-α production, which in turn activates autoractive Th1 and Th17 cells to produce more inflammatory cytokines and feed in to the cycle of LL37 production. (Adapted from [457])

sequences [452]. A NF-κB binding site has been identified in the human cathelicidin promoter [453].

II.6.4.2 Acne rosacea

On the contrary, abnormally high levels of LL37 are found in the inflammatory skin condition called acne rosacea, which is characterized by erythema (redness) caused by dilated blood vessels across the central portion of the face. Usually, LL37 secreted onto the skin surface is further processed into smaller peptides, which maintain the antimicrobial activity but are less immunogenic [256] (Figure 21). In this condition posttranslational processing abnormalities lead to the occurrence of

different proteolytically processed forms of cathelicidin peptides in patients as compared to healthy individuals [454]. This is associated with an increase in stratum corneum tryptic enzyme (SCTE), which is the key protease that cleaves hCAP18 to active peptides in the epidermis. Yamasaki *et al.* could show that injection of LL37 into the skin caused erythema and vascular dilation, together with the influx of inflammatory cells, thereby showing causality for overexpressed LL37 levels in the disease condition. Additionally, the expression profile of LL37 may influence the local microibiota population, which may in turn contribute to the inflammatory response.

II.6.4.3 Psoriasis

Furthermore, LL37 was shown to be involved in the autoimmune inflammatory skin disorder psoriasis. In this condition, recurrent inflammation is driven by association of LL37 with self-DNA into aggregated and condensed structures that are delivered to the early endocytic compartments of plasmocytoid DCs (pDCs) where they are recognized by TLR9 [455] (Figure 30). pDCs are normally sensors for viral or microbial DNA, but inert to self-DNA. Why the combination of self-DNA and LL37 overcomes this restriction and cause autoimmune disease is yet poorly understood [456]. As a response pDCs release Type I interferons, which trigger a cascade of myeloid DC maturation and activation of autoreactive Th1 and Th17 cells. These T cells produce cytokines such as interferon-γ, IL22 and IL17, which in return activate LL37 expression and thereby feed into the inflammatory cycle.

II.6.5 The role of AMPs in disease of the oral cavity

We can find members of prominent AMPs families in the oral cavity: defensins, LL37 and histatins. The concentration of defensins in saliva and oral tissue is linked to copy number variations and correlations with the susceptibility to caries have been investigated. Levels of HNP1-3 are much lower in saliva of children displaying caries than in healthy controls [458]. Deficiency in oral AMPs for example is linked to the congenital Kostmann syndrome. Patients suffer from severe neutropenia, causing recurrent oral infections and periodontal disease. The low numbers of neutrophils in Kostmann syndrome patients show diminished concentrations of cathelicidin and α-defensins [459].

II.6.6 The role of AMPs in systemic infections

As mentioned before AMPs are expressed all throughout the human body and by a broad variety of cells. The diversity of their structures and expression patterns highlights their importance in the defense of the organism, not only on the surfaces facing the environmental challenges, but also in the case of systemic threats, such as HIV or sepsis.

II.6.6.1 HIV infection

The activity of AMPs towards viruses is not discussed extensively in this thesis. Nonetheless, the effectiveness of defensins in the defense against HIV can not be neglected [460]. A study in human oral epithelial cells showed HIV-induced expression of DEFB2 and DEFB3 mRNA. The protective effect of these defensins was shown by inhibition of virus replication and failure of HIV to infect target cells. Two mechanisms for HIV inhibition have been proposed. One of them is binding of defensins to viral particles, which was confirmed by electron microscopy. Additionally, defensins induced downregulation of the HIV coreceptor CXCR4 in peripheral blood mononuclear cells and T cells, thereby inhibition entry of the virus [461]. These results underline the importance of AMPs in mucosal protection against HIV infection and open up questions about susceptibility, polymorphisms, and variations of copy numbers.

Two studies in perinatally HIV infected children in Italy and Brazil reported a significant association between a SNP in the 5'-untranslated region of the DEFB1 gene and infection if born to seropositive mothers, underlining the role of defensins in

systemic protection [462,463]. A later study in Brazilian children associated lower copy numbers of the DEFB4 encoding gene in HIV-positive subjects to susceptibility of infection [464]. DEFB4 is expressed in the female reproductive tract and therefore could be involved in protection from vertical transmission.

The HIV-protective capacities of α-defensins were suggested by a study in continuous exposed but seronegativ women. Their expression of HNP1-3 by peripheral and mucosal $CD8^+$ T cells was 10-fold higher than in control subjects and showed a strong antiviral activity *in vitro* [465]. Moreover, another study suggested a protective role for increased α-defensin titers in breast milk, in relation to mother-to-child HIV transmission [466].

II.6.6.2 Sepsis

Sepsis is the systemic inflammatory response towards infection. Its outcome can be grave when cell death leads to tissue damage, multiple organ failure, septic shock and death [467]. A presented case-control study in a Chinese Han population deciphered the associated between SNPs in the DEFB1-encoding gene and severe sepsis [468]. They identified various haplotypes with differential phenotypic outcomes: the -44G/C variation was associated with both the susceptibility to and the fatal outcome of severe sepsis, the -20G/-44G/-52G haplotype was associated with the fatal outcome of severe sepsis, whereas the -20A/-44C/-52G haplotype showed a protective role against severe sepsis.

II.7 Induction of AMPs expression by diverse stimuli

The general expression of AMPs is first of all regulated according to a developmental schedule. This has been observed in the mouse model as well as in humans. Then, the repertoire of inducible AMPs can be regulated by various stimuli, coming from the host itself, such as cytokines and hormones, or by external signals, such as microbes or dietary compounds. Furthermore, the amount of expressed peptide can be regulated on the posttranscriptional level. Because the activity of AMPs relies on proteolytic processing of the pro-peptide, it is correlated to the expression and activity of certain proteases. The following chapter will summarize the known stimuli that

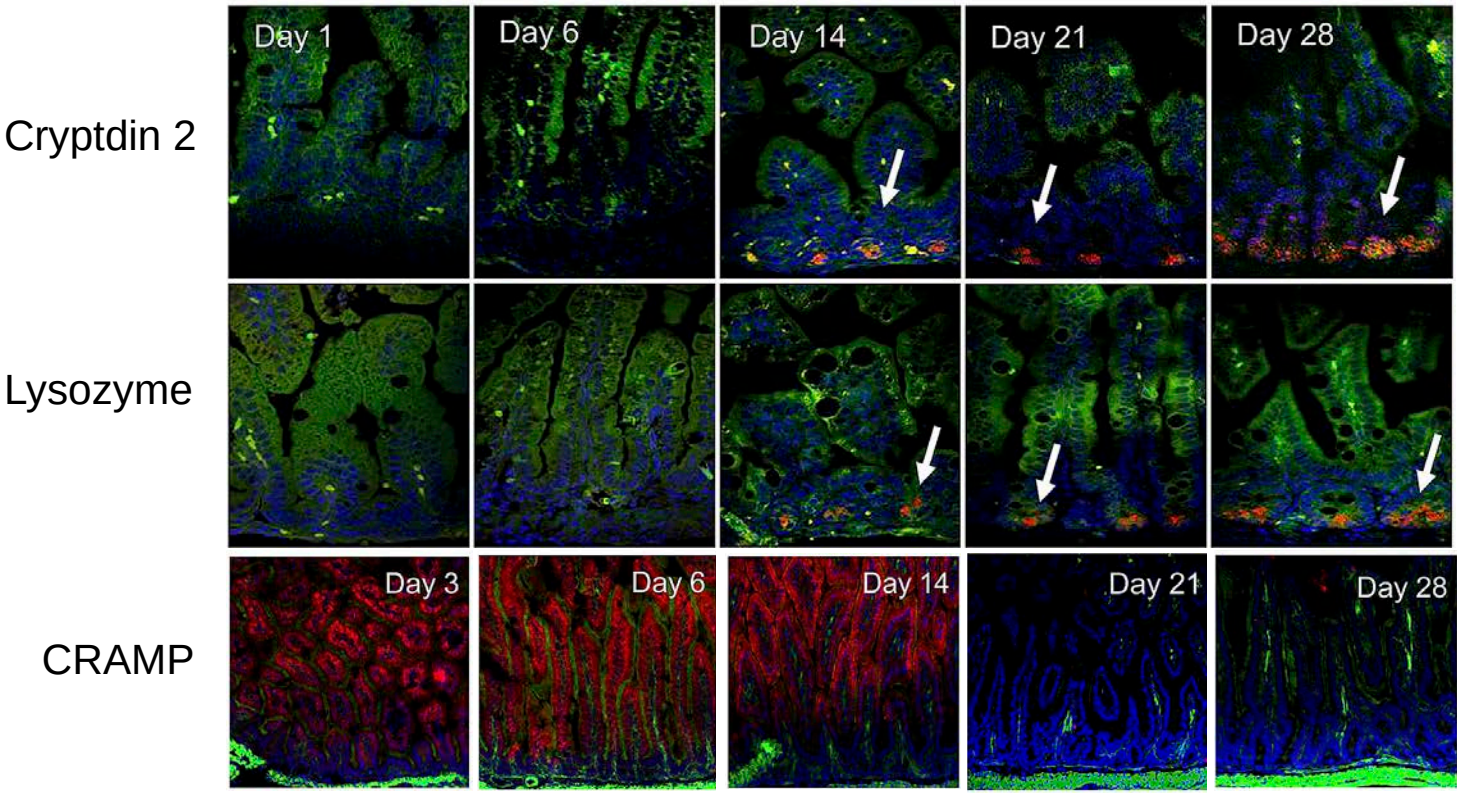


Figure 31: Developmental induction of AMPs in new born mice. The expression of AMPs in the intestine of mice follows a developmental program. The induction of expression of some AMPs, such as cryptdin 2 and lysozyme, occurs approximately 2 weeks after birth (upper two panels). On the contrary, some AMPs, such as CRAMP are expressed already in the neonate, but then disappear between 2 and 3 weeks of age (lower panel). (Adapted from [470])

have been described to induce or suppress the expression of AMPs in the human body, but also in other species.

II.7.1 Host-derived inducers of AMPs expression

First, we will focus on the host-induced expression of AMPs, discussing the developmental regulation of AMPs expression during the pre- and post-natal phase in the mouse model as well as in humans. Furthermore, internal host-derived AMPs-inducing molecules, such as hormones and cytokines, will be discussed in this chapter. Hormones take a special place in host-derived regulation of AMP expression, as they can induce, as well as inhibit their expression. In this regard stress hormones will be discussed as repressors of AMP expression.

II.7.1.1 Developmental induction of AMPs expression

The development of the immune system of the fetus is by far not complete at the time of birth. The transition from the mother's womb into the open air goes along with a huge immunological challenge posed by the microbial environment. Several pathogens have been described to cause severe neonatal disease and mortality through infection via the transplacental route during pregnancy, or transmission from the mother to the newborn during passage through the birth canal [469]. In these scenarios the expression of innate defense molecules such as AMPs are of major importance in the immunologically underdeveloped fetus or neonate. Only few studies have been undertaken to determine the developmental onset of expression of AMPs in neonates in humans and in the mouse model. All of them suggest a developmental regulation of AMP expression in various tissues. *In vivo* studies of the neonatal mouse small intestinal epithelium have shown that the first two weeks after birth are dominated by high constitutive expression of CRAMP (Figure 31). This conferred protection from the enteric pathogen *L. monocytogenes* [470]. Interestingly, CRAMP expression gradually declines and is absent in the small intestine in adults. Decline of CRAMP expression goes hand in hand with increased stem cell proliferation and the formation of intestinal crypts. Paneth cells take over the production of AMPs, such as cryptdins after a period of 2 weeks [471,472] (Figure 31). Developmental regulation is partly mediated by enhanced Wnt signaling which accompanies increased epithelial proliferation during the postnatal phase [473].

Compared to the postnatal establishment of AMP defense in mice, human enteric AMP expression starts already during late gestation as observed in a study of human fetal samples [474]. Quantifiable levels of HD5 and HD6 mRNA accumulate in the small intestine towards the end of the second trimester. Their expression was localized to Paneth cells, which appear at 12 weeks of gestation in humans. But levels were found to be 40-250-fold lower than those observed in adults. This can be explained by the much lower ratio of Paneth cells in the fetal crypts, as compared with the newborn or adult.

Developmental regulation in expression was also observed in other tissues. A study of AMP expression in the neonatal skin found significantly elevated expression of cathelicidins CRAMP in the skin of newborn mice and LL37 in foreskin of human neonates when compared with adult skin [475]. In the neonatal lung DEFB2 was found to be the most prevalent AMP and its expression was less abundant in premature compared with term or post-term tissue [476].

Interestingly, there are several AMPs based mechanism of protection for the fetus and newborn. For example the amniotic fluid contains HNP1 to 3, lysozyme and LL37 [477,478]. Additionally, the vernix caseosa, which is a lipid-rich white substance that covers the skin of the fetus and the newborn, contains HNP1 to 3, lysozyme, LL37, and psoriasin. Therefore these two structures protect the fetus within the womb. After birth AMPs contained in the breast milk, such as lactoferrin, lysozyme, and DEFB1, supplement AMPs protection [479,480]. Higher DEFB1 immunoreactivity was demonstrated in breast tissue during lactation.

II.7.1.2 Induction of AMPs expression by cytokines

Cytokines are produced by a wide range of cells, including immune cells and epithelial cells and function as important signaling molecules in cellular communication. Their production is highly increased in response to an infectious agent or inflammatory stimulus and it is not surprising that they are involved in the induction of AMPs expression [481] (Figure 32).

II.7.1.2.1 Members of the IL1 family

Pro-inflammatory cytokines, such as the IL1 family members IL1α, IL1β and IL18, are key inducers of AMPs expression. They are expressed by a number of immune cells

but also by epithelial cells. Their target receptor is type I IL1 receptor, which signals downstream via the adaptor molecules MYD88 and IRAK4 and activates several transcription factors, such as NF-κB, AP1, JNK and p38 [482]. Expression of DEFB2 in the human colon epithelial cell lines HT-29 and Caco-2 is rapidly induced by IL1α stimulation [280]. Furthermore, IL1α increased expression of skin AMPs, such as lipocalin 2, S100A8, S100A9 and SLPI in keratinocytes [483,484]. In the oral cavity IL1α up-regulated expression of S100A7 and S100A12 in gingival keratinocytes [485]. Moreover, a study by Moon *et al.* showed IL1α-induced upregulation of DEFB2 in human middle ear epithelial cells [486].

Nothing has been described about the involvement of IL1β in intestinal AMPs expression so far. But it was shown to induce the expression of DEFB2 in bronchial, tracheal and nasal airway epithelia [286,487,488], as well as human astrocyte cultures [489]. Furthermore, a co-culture study by Pioli and colleagues found that IL1β from blood monocytes and uterine macrophages induces secretion DEFB2 by uterine epithelial cells [490].

Also IL18 was found to induce expression of LL37 and DEFB2 in intestinal epithelial cells [491].

II.7.1.2.2 Th17 cytokines – IL17 and IL22

Th17 cells are a distinct lineage of effector $CD4^+$ T cells. Their differentiation from naïve T cells via TGFβ is initiated by proinflammatory cytokines, such as IL6, IL1β and TNFα [492]. After, the maintenance and survival of Th17 cells is dependent on IL23. They produce the cytokines IL17 and IL22 [493]. Several studies indicate that Th17-cell-derived cytokines act as key regulators of the AMP response *in vitro* and *in vivo* (Figure 31). IL22 is an important player in the maintenance of mucosal homeostasis. An *in vivo* study in mice showed that IL22 was required for the induction of expression of the C-type lectins REG3β and REG3γ in colonic epithelial cells in the context of *Citrobacter rodentium* infection. IL22-deficient mice were highly susceptible to infection with this murine enteropathogen, as shown by increased tissue damage, systemic bacterial burden and mortality. Administration of exogenous REG3γ was able to improves survival in this setting [66]. Another study in a mouse model of dextran sodium sulfate-induced acute colitis showed the importance of

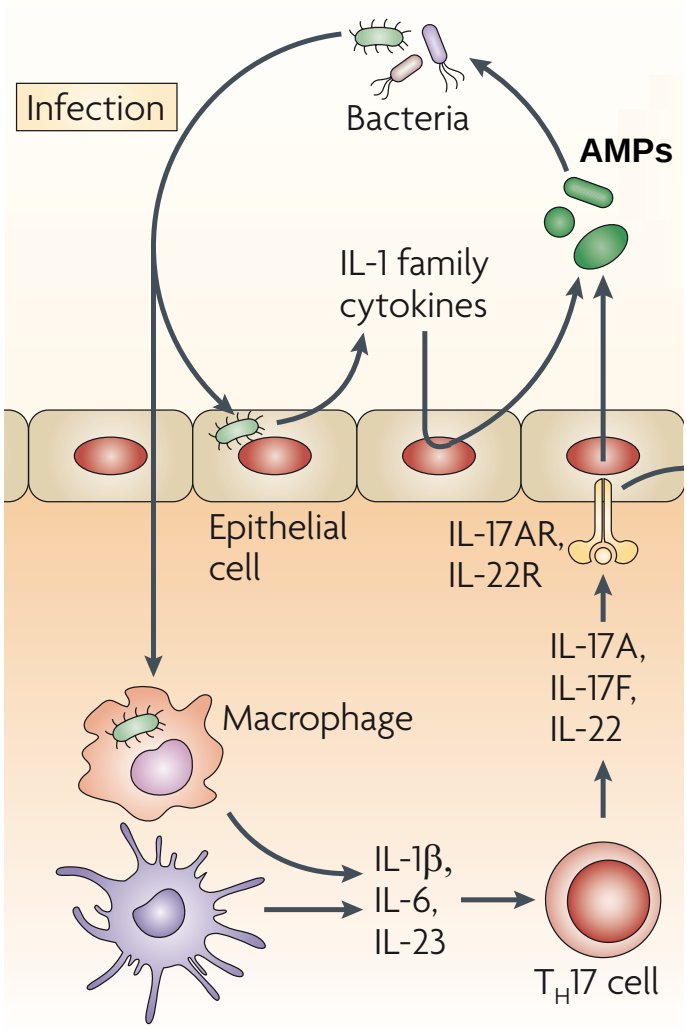


Figure 32: Induction of AMP expression by cytokines. Members of the IL1 family and Th17 cell-derived cytokines are important host-derived inducers of AMP expression. Bacterial infection, for example, can induce the expression of IL1β by epithelial cells and macrophages and DCs. In turn, this cytokine mediates the expression of AMPs by epithelial cells, and together with IL6 and IL23, released from macrophages and DCs, induces the differentiation of Th17 cells. Those cells then produce IL17A, IL17F and IL22, which further induce AMP expression by the epithelium. (Adapted from [481])

recruitment of neutrophils upon tissue damage. These neutrophils expressed high levels of IL22 in an IL23-dependend manner and thereby induced the upregulation of REG3β and S100A8 in colonic epithelial cells. The protective effects of neutrophils in this setting was shown by the fact that transfer of IL22–competent neutrophils to *Il22a*-deficient mice protected the colonic epithelium from dextran sodium sulfate-induced damage [494].

Several studies describe the role of Th17 cytokines in other tissues. Combination of IL17 and IL22 induced the expression of DEFB2, S100A9, S100A7 and S100A8 in primary keratinocytes [493]. Additionally, IL17 was shown to synergistically increased the vitamin D-induced expression of LL37 in keratinocytes [495]. This mechanism could have implications in psoriasis development, where Th17 cells are prominent. Lai and colleagues could show that IL17A can also induce the expression of REG3α in human keratinocytes and that REG3α can act on keratinocytes to augment their proliferation [496]. The authors suggest implications in psoriasis and wound repair. A study by Guilloteau and colleagues used a cocktail of IL17, IL22 with IL1α, TNFα and oncostatin M to induce upregulation of DEFB2, S100A7 in human skin explants. *In vivo,* intradermal injection of these five cytokines induced expression of 12 different AMPs in the skin of mice [484]. Furthermore, IL17 was able to stimulate DEFB2 expression >75 fold in primary human airway epithelial cells [497,498].

II.7.1.2.3 TNFα

The pro-inflammatory cytokine TNFα seems to be only a weak inducer of DEFB2 in the human intestine [280] but is capable to stimulate AMPs release in other tissues. TNFα was shown to induce expression of DEFB2, DEFB3 and DEFB4 in human gingival epithelial cells [499,500] and DEFB2 in human lung epithelial cells [501], corneal epithelial cells [502] and astrocyte cultures [489]. Furthermore, it stimulated expression of lingual antimicrobial peptide, a member of the β-defensin family of peptides, in cultured bovine tracheal epithelial cells [503].

II.7.1.3 Induction of AMPs expression by estrogen

Estrogens are important regulators of female physiology. Recent epidemiological data suggest a correlation between decrease of estrogen levels after menopause and increased susceptibility to urinary tract infections [504]. A study in pre- and

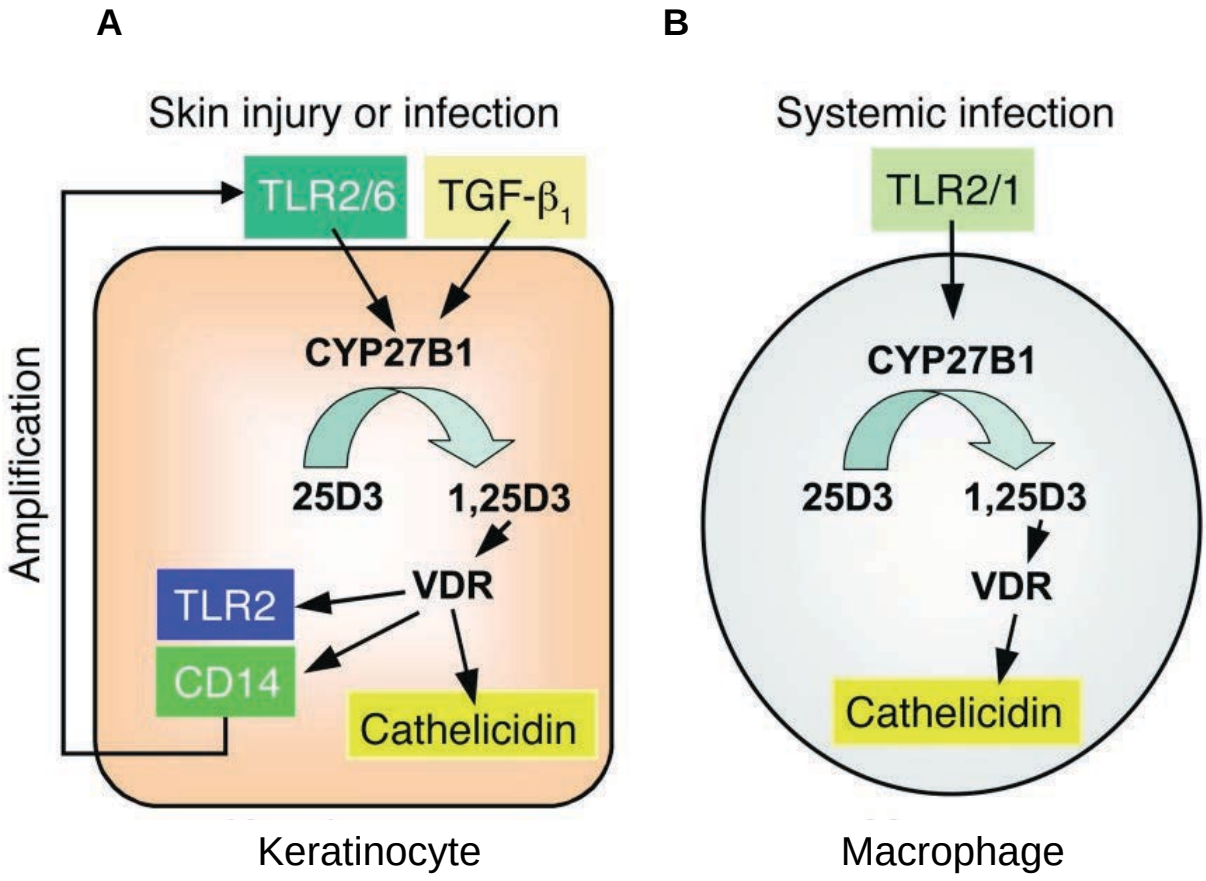


Figure 33: Regulation of cathelicidin expression by vitamin D. The expression of cathelicidin is regulated by two distinct 1,25D3-dependent pathways in keratinocytes and monocytes. In the skin, keratinocytes are activated upon injury by TGFβ or TLR2/6 ligands, which then leads to induction of CYP27B1. As a consequence 25D3 is converted to 1,25D3, which binds to the VDR and induces cathelicidin, TLR2, and CD14 (A). The thereby increased TLR2 expression acts as a positive feedback for further keratinocytes activation and cathelicidin expression. In contrast, circulating macrophages are activated by TLR2/1 agonists, which induce the expression of the VDR and CYP27B1 (B). In consequence, CYP27B1 converts 25D3 to 1,25D3 and subsequently increases cathelicidin. (Adapted from [512])

postmenopausal women investigated the role of estradiol in urinary AMPs expression [505]. A 2-week supplementation of estrogen significantly increased the expression of AMPs, such as DEFB1, DEFB2, psoriasin, RNase 7, and cathelicidin, in urinary epithelial cells. Additionally, estrogen promoted the expression of cell-cell contact–associated proteins, thereby strengthening the epithelial integrity and barrier function. Another study showed that estradiol stimulates a five fold increase in DEFB2 expression in human uterine epithelial cells [506]. Responsiveness of tissues to estrogen is mediated by two estrogen receptors (ER), ERa and ERb, and by the estrogen-responsive elements (ERE) located in the promoter regions of target genes. EREs have been reported in the promoters for DEFB1, DEFB2, and psoriasin [507].

II.7.1.4 Induction of AMPs expression by vitamin D

A special case of induction is described for LL37. Its promoter contains a vitamin D responsive element (VDRE). This consensus sequence can be bound by the vitamin D receptor, which is thereby able to act as a transcription factor. Activation of this process in achieved by its ligand 1,25-dihydroxyvitamin D_3 (1,25D3), the hormonal form of vitamin D_3. Binding of ligand-stimulated vitamin D receptor was shown to induce LL37 expression in human keratinocytes, monocytes and neutrophils [508,509] and *in vivo* in human skin [510] (Figure 33). The induction of LL37 by vitamin D in intestinal epithelial cells has not been documented.

Vitamin D_3 is produced in the skin from the cholesterol precursor 7-dehydrocholesterol under the influence of UVB light [511]. The activation of vitamin D_3 to 1,25D3 requires 2 major hydroxylation steps, the first by 25-hydroxylase (CYP27A1) and the second by 1α-hydroxylase (CYP27B1). A negative feedback mechanism initializes the induction of 24-hydroxylase (CYP24A1) and degradation of 1,25D3 to limit the level of this bioactive hormone.

Recent studies investigated the relations between infection, injury and vitamin D induced LL37 expression. They were able to demonstrate that stimulation of TLRs on human macrophages induces CYP27B1 for the conversion of 25D3 to active 1,25D3 and increases the expression of the vitamin D receptor (VDR) for the activation of LL37 expression [442] (Figure 33 B). These results indicate a link between infection and the activation of LL37 expression via vitamin D. A follow up study in

keratinocytes showed that injury is able to induce CYP27B1 and thereby increase levels of 1,25D3 in the skin (Figure 33 A). Additionally, factors involved in

the wound repair such as TGF-β induced 1,25D3-dependent immune responses. Furthermore, they could show a 1,25D3-dependent increase of TLR2 and CD14 receptors. These findings describe a sort of feedback loop which allows the activation of AMP immune responses upon injury and at the same time increases the level of detection of microbes and thereby responsiveness in the skin immune system in a vitamin D dependent manner [512].

II.7.1.5 Stress hormones as repressor of AMPs expression

Sustained stress is a common health threat in our society nowadays and negatively influences various physiological processes [513]. Also the immune system is under the control of the stress response. While acute stress is thought to be immunoenhancing in scope of the “fight-and-flight” syndrome, sustained stress leads to the impairment of effective immune responses. Stress can be communicated via three major pathways: The catecholamine pathway via adrenergic stimulation, the glucocorticoid pathways via activation of the hypothalamic-pituitary-adrenal axis, and the cholinergic pathway via acetylcholine. Several studies have been performed to shed light on the influences of stress onto AMPs expression and evidence for the involvement of each of these pathways has been described. No intestine-specific studies have been undertaken so far.

II.7.1.5.1 Glucocorticoids

Glucocorticoids are steroid hormones expressed by the adrenal cortex and cortisol is the best-known member of this family. They signal via the glucocorticoid receptor, which can directly act as a transcription factor. Their anti-inflammatory capacities are well described and they are widely used in medicine to treat inflammatory disorders. A study in human primary bronchial epithelial cells showed the inhibition of inducible DEFB3 after pretreatment with glucocorticoids before infection with heat-killed *P. aeruginosa* [514]. Also the expression of skin antimicrobial peptides in the frog *Rana esculenta* was completely blocked after administration of glucocorticoids [515].This went hand in hand with an increase of viable mouth bacteria. A mechanism where

glucocorticoids induce the NF-κB inhibitor IκBα to block AMPs gene transcription was presumed in this scenario.

II.7.1.5.2 Cholinergic stimulation

Cholinergic receptors are ligand-gated ion channels, which are activated by the neurotransmitter acetylcholine. Additionally, the nicotinic acetylcholine receptor (nAchR) can be activated by nicotine and the muscarinic acetylcholine receptor (mAchR) can be activated by muscarine. Cholinergic activation was shown to inhibit local proinflammatory responses and anti-inflammatory effects on immune cells and epithelia have been studied [516-518]. Activation of the nAchR by topic nicotine administration reduced AMPs activity in the skin of mice and increased their susceptibility to methicillin-resistant *S. aureus* infection [519]. These results demonstrate possible mechanisms for the increased risk for infections followed prolonged stress or nicotine exposure, which both activate the nAchR pathway.

II.7.1.5.3 Physiological stress

One group investigated the effect of physiological stress, such as combined insomnia, noise, and crowding, in a mouse model of cutaneous infection [520]. Cutaneous barrier function was severely decreased by physiological stress as well as administration of endogenous glucocorticoids. Both CRAMP and mBD3 expression was significantly reduced, which lead to increased severity of group A *Streptococcus pyogenes* skin infection. They hypothesized that an inhibition in epidermal lipid synthesis decreased the secretion of lamellar bodies, which are important carriers for skin AMPs also in humans. The effects could be reversed by pharmacological blockade of stress hormone action, as well as topical administration of physiologic lipids.

The response to stress in cattle and the relationship to pneumonia has also been investigated. Transportation, weaning, and commingling results in elevated levels of stress hormones, such as glucocorticoids and catecholamines. Results from a study by Mitchell *et al.* indicate that glucocorticoids inhibited expression of AMPs such as tracheal antimicrobial peptide and lingual antimicrobial peptide in bovine tracheal epithelial cells [521].

II.7.1.5.4 Physical stress

Two human *in vivo* studies proclaimed a reduction in innate immune responses in elite athletes by physical stress, signaling via the HPA axis and cortisol release. They reported reduced levels of LL37 and DEFB2 in saliva from individuals and this correlated with the occurrence of upper respiratory tract infections [522,523].

II.7.2 Induction of AMPs expression by external stimuli

Various external stimuli have been described to induce the expression of AMPs. Among them are bacteria and bacterial products as well as dietary compounds, which will be discussed in the following chapter.

II.7.2.1 Induction of AMPs by bacteria

Bacterial stimuli are important for the development of our immune system as described in chapter I. Germ-free mice are a useful tool to study the overall role of microbial signals in AMPs expression. Some intestinal AMPs are expressed independently of the microbiota, such as lysozyme or PLA2s. Others require bacterial signals for their expression and are absent in germ-free mice, such as members of the cryptdins-related sequence family of peptides, angiogenin 4 (ANG4) and REG3γ [247,524,525].

In an infectious setting AMPs have been shown to be inducible by several gram-positive and gram-negative bacteria. DEFB2 expression in human colon epithelial cell lines is rapidly induced by infection with enteroinvasive bacteria, such as *Salmonella* and *E. coli* species [280]. In the same study human fetal intestinal xenografts were used to how DEFB2 induction by intraluminal infection with *Salmonella*.

Induction of AMPs expression by bacteria has also been described in other tissues. *P. aeruginosa* infection for example stimulated DEFB2 expression in A549 lung epithelial cell line [286]. Infection of keratinocytes with *S. aureus* upregulated expression of DEFB2, DEFB3 and LL37 [526]. In gastric epithelial cells infection with *H. pylori* induced expression of DEFB2. In accordance an increased DEFB2 level was also found in biopsies from patients with *H. pylori*-positive gastritis [527].

Also, bacterial molecules alone are able to induce expression of AMPs via innate receptor signaling pathways. Two different studies described the ability of flagella filament structural protein (FliC) in the supernatant of *Salmonella* cultures to induce DEFB2 expression in the intestinal epithelial cell line Caco-2 cells in a NF-κB dependent manner [528,529]. Vora and colleagues showed that LPS and peptidoglycan stimulate DEFB2 expression in a TLR4- and TLR2/TLR6-dependent manner in intestinal epithelial cells [530]. Furthermore, LPS was found to induce DEFB2 in human astrocyte cultures [489]. Moreover, several studies reported LPS and LTA mediated activation of DEFB2 expression in airway epithelial cells via TLR4 and TLR2 respectively [531-533].

Activation of TLRs converges in the signal cascades of MAP kinases and NF-κB. The role of these two pathways in AMPs expression will be discussed in greater depth in the following chapters.

II.7.2.2 Induction of AMPs expression by SCFA

SCFA are produced in vast amounts by fermentation processes of the intestinal microbiota. Their involvement in several gut physiological processes has been described in chapter I. Several studies demonstrated their immune modulating role via the induction of cathelicidin in the intestine. Cell differentiation seems to be a key determinant of LL37 expression, as observed in human colonic biopsies [260,534]. *In vitro* studies in human colonic cell lines identified butyrate, isobutyrate and propionate as inducers of cell differentiation and LL37 expression [534]. The use of specific inhibitors showed the involvement of several signaling pathways in the butyrate-mediated expression of LL37, such as the vitamin D pathway, MAPK pathways and TGFβ signaling [534,535]. Although it seems like different MAP kinases are involved in different colonic cell lines and other cell types such as lung epithelial cells and keratinocytes [536,537]. *In vivo* studies of a rabbit model of *Shigella* infection showed that butyrate treatment significantly increased the expression levels of the rabbit homologue to LL37, CAP18 (Figure 34). This lead also to reduction in the bacterial load and amelioration of clinical symptoms and inflammation in the colon [264]. Furthermore, treatment with the butyrate stimulated DEFB2 expression in colonic epithelial cells [538].

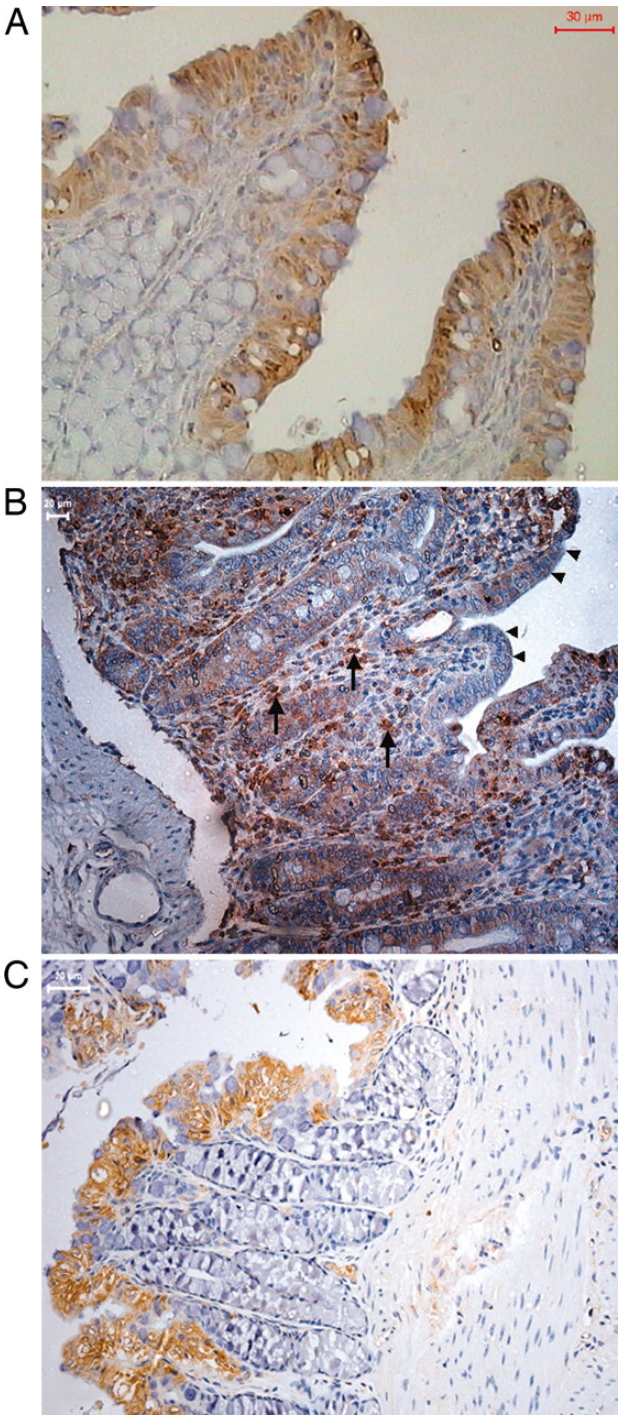


Figure 34: Colonic expression of the rabbit cathelicidin CAP18 upon *Shigella* infection and SCFA treatment. Imunohistochemical staining showed localization of CAP18 (brown) almost exclusively located in the surface epithelium in healthy rabbits (A). On the contrary, *Shigella*-infection led to the disappearance of CAP18 on the surface and in crypts (arrowheads) (B). On the other hand, CAP18 staining was increased in inflammatory cells in the lamina propria upon infection (arrows). Treatment of infected rabbits with butyrate lead to the reappearance of CAP18 staining in the surface epithelium and disappearance in the lamina propria [264].

II.7.2.3 Induction of AMPs expression by the amino acid isoleucine

The essential amino acid isoleucine has been described in one study as an inducer of β-defensins in bovine kidney epithelial cells in a NF-κB dependent manner [539].

Summary

In Chapter II, we have discussed the existence of AMPs throughout the kingdoms of life, their characteristics and properties. We have learned that they are not only capable of killing a multitude of microbes, but also important in the activation of the adaptive immune response, tissue repair, and restitution. In a bigger picture, we have discussed the involvement of AMPs in human pathologies and the detrimental effects of the deregulation of their expression. Their importance for intestinal homeostasis is indisputable, as illustrated on the example of IBDs. As we learn more about these multitalented peptides we wonder how we can manipulate their expression? In this regard, we have discussed their inducibility by host molecules, as well as bacterial factors and nutritional components. To go deeper into the regulation of inducible AMPs expression we will take a look on the basics of genomic organization and general gene expression. Different mechanisms, which allow for rapid and distinguished stimulus-induced gene expression, will be discussed. Following this part of general inducible gene expression we will take a look on what is known about the regulation of AMPs gene expression by transcription factors and epigenetic mechanism. In this regard, we will focus on the role of acetylation of histone tails in gene expression, and we will learn about enzymes, which are involved in acetylation and deacetylation of histones and other proteins.

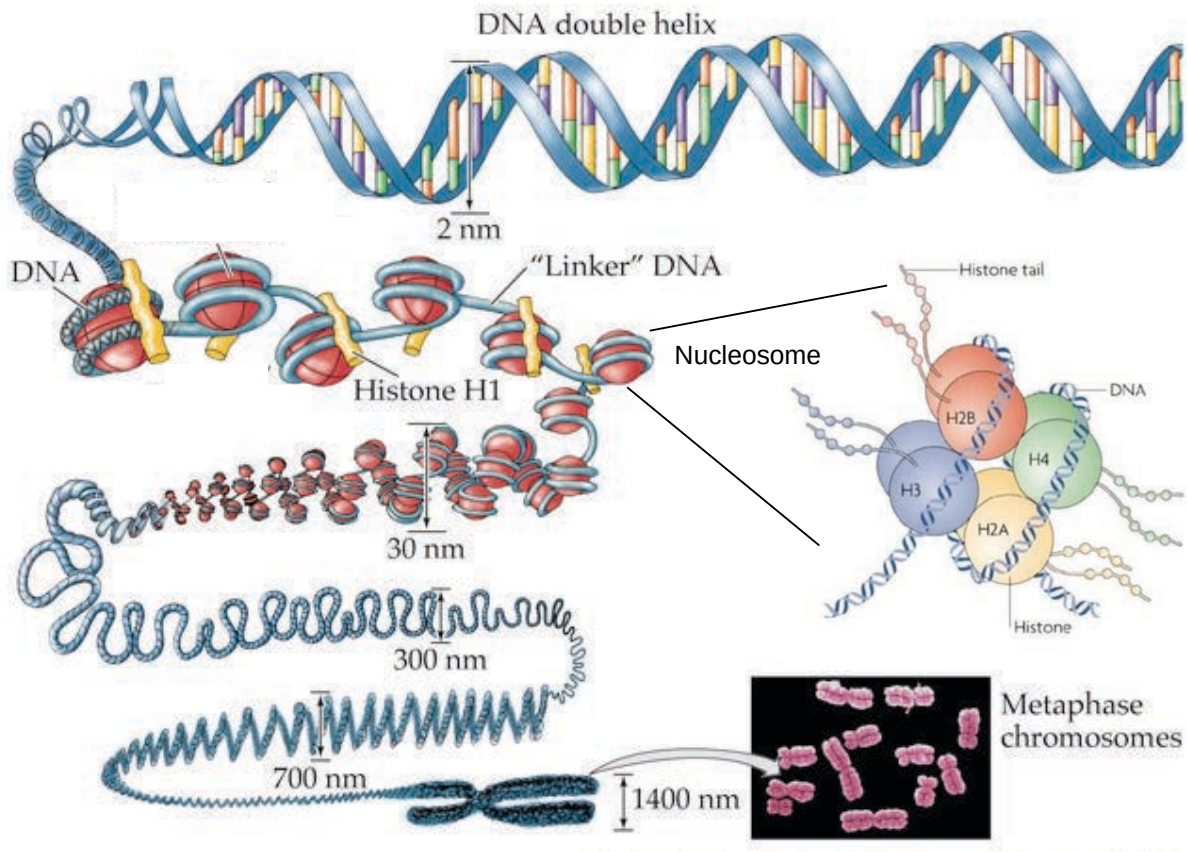


Figure 35: Structural organization of DNA. Each cell contains double stranded DNA of a total length of 2 meters. In order to fit this length into the nucleus it needs to be tightly packed into a structure called chromatin. This structure consists of so-called nucleosomes, which contain a complex of eight core histone molecules wrapped with 147 base pairs of 1.7 turns of DNA. Additionally, histone H1 clamps the DNA to the histone core, supports the next level of packaging into highly condensed fibers. (Adapted from "Life: The Science of Biology, 7th Edition, 2003" and [540])

Chapter III – Regulation of inducible gene expression

As discussed in chapter II, AMPs genes can be either expressed constitutively or induced by a wide variety of host-derived or external stimuli. Deciphering the mechanism, which govern the inducibility of certain AMPs is important to understand the basis of their regulation. Also the question, whether AMPs and INFs are regulated in the same ways in response to external stimuli is intriguing. In order to go deeper into the stimulus-induced expression of AMPs, we have to understand the basic mechanisms, which allow inducible gene expression. External signals such as growth factors, stresses, cytokines or pharmacological agents activate pathways, which lead to the rapid expression of primary response genes. Those are specified as being directly connected to the signaling machinery and do not require protein synthesis for their expression [541]. To understand the regulation of expression of inducible genes, it is important to consider the organization of the human genome into chromatin. The following chapters are aiming to describe the molecular organization of DNA in eukaryotic cells and the complex mechanisms, which regulate accessibility of the stored genetic information.

III.1 Structural organization of the human genome

The human genome contains around 20 000 protein-coding genes, encoded in a total length of over 3 billion base pairs of DNA [542]. In order to be packed into the confined space of the nucleus of eukaryotic cells, the DNA double strand is highly compacted into a structure called chromatin [543] (Figure 36). But this compacted structure is still very dynamic to allow for important cellular processes such as transcription, repair, or cell division. The packing of DNA is achieved by wrapping of a piece of 147 nucleotide pairs (1.7 turns) around an octamer of core histone proteins (2 copies each of histones H2A, H2B, H3, and H4) to form a so-called nucleosome [544,545] (Figure 35). This basic unit of chromatin is repeated every 200 base pairs and further stabilized and compacted by histone H1. In mitosis, the chromatin reaches its highest level of condensation into the chromosomes, which are then ready for cell division. During interphase, the DNA exists in two chromatin states of different levels of compaction.

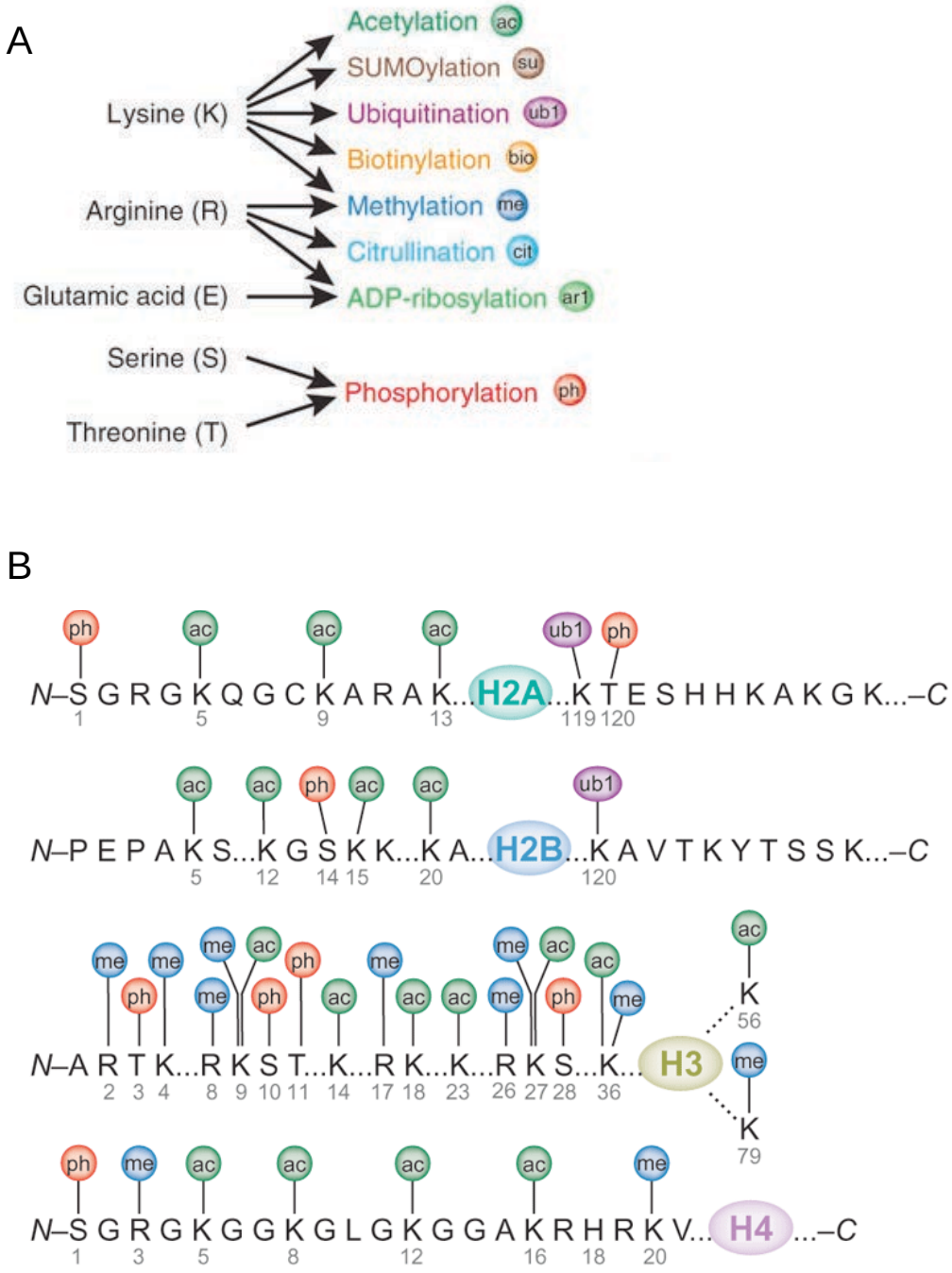


Figure 36: Post-translational modifications of histone proteins. Known post-translational modifications and the amino acid residues they modify (A). The scheme presents most common histone modifications include acetylation (ac), methylation (me), phosphorylation (ph) and ubiquitination (ub1) (B). Most of the known histone modifications occur on the N-terminal tails of histones, with some exceptions including ubiquitination of the C-terminal tails of H2A and H2B and acetylation and methylation of the globular domain of H3 at K56 and K79, respectively. Globular

domains of each core histone are represented as colored ovals. (Adapted from [546] and [547])

Heterochromatin is the denser of the two structures and is generally associated with inactive gene expression. This compact structure can generally be found at the centromere and telomeres of the chromosome. The structure of euchromatin in contrast is much more relaxed and contains most of the active genes.

III.2 The role of chromatin in gene accessibility

As mentioned previously, the compacted structure of DNA still has to be highly flexible and is constantly exposed to remodeling processes in order to allow gene expression, DNA replication, repair or recombination. Remodeling is orchestrated by chromatin-remodeling complexes [548,549]. Facilitated by the use of energy from ATP hydrolysis, nucleosomes can be repositioned, replaced by histone-variants or expelled to loosen up the chromatin structure and increase accessibility. So far, five families of chromatin remodelers have been identified in eukaryotes: SWI/SNF (SWItch/Sucrose NonFermentable), ISWI (Imitation SWI), Mi-2/NuRD (nucleosome remodeling deacetylase) complex, INO80 and SWR1. They all possess a common ATPase domain. But each remodeler complex has unique protein domains and consists of different subunits possessing histone-modifying capacities. Therefore, their functions can be variable and either activating or repressive. In order to recruit these complexes to target genes, specific signals are set for the recognition by interacting domains. These signals occur in the form of post-translational modification marks on core histones, which are set by a large variety of histone-modifying enzymes.

III.2.1 Histone modification

The N-terminal tails of core histones protrude from the nucleosome structure and are subjected to a vast array of covalent post-translational modifications (Figure 35 and 36). Among them are mono-, di-, or trimethylation of lysine, mono- or dimethylation of arginine, acetylation of lysine, phosphorylation of serine, threonine or tyrosine, ubiquitylation of lysine, sumoylation of lysine, and many more [550] (Figure 36). Occurrence of these modifications has been extensively mapped in yeast [551], plants [552], drosophila [553], nematodes [554], humans [555] and a variety of human cell lines [557].

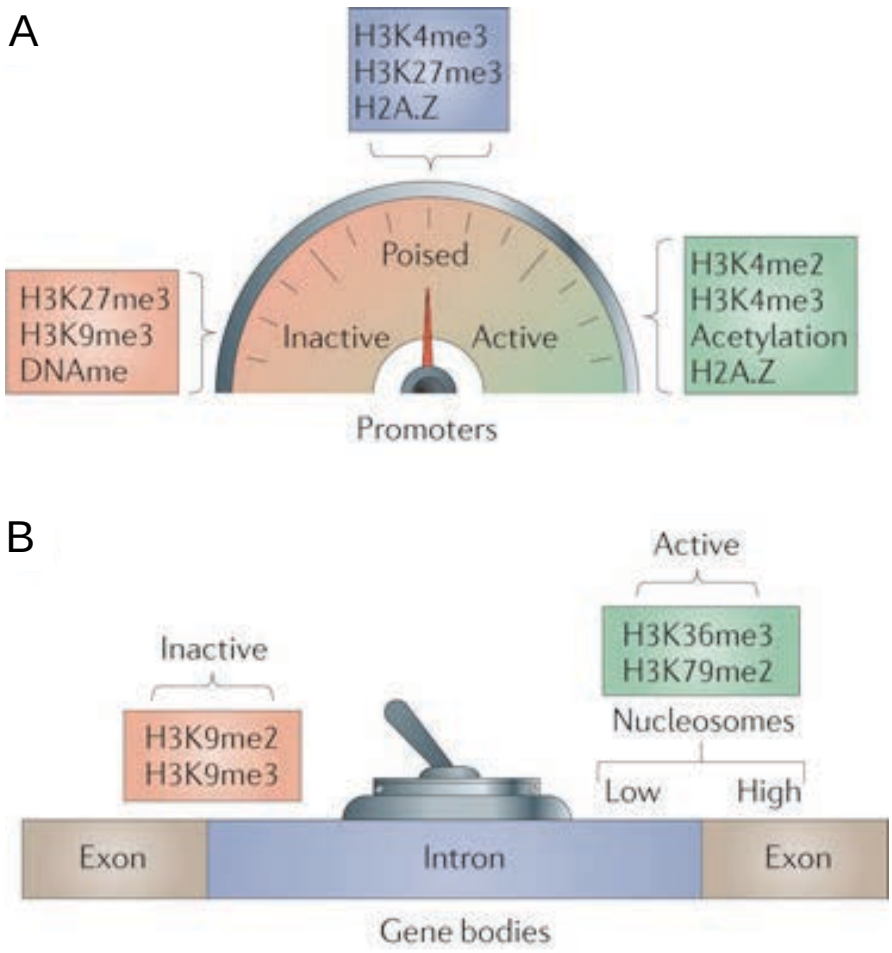


Figure 37: Characteristic chromatin marks of promoters and gene bodies of active or inactive genes. Histone modifications may serve as 'switches' as promoters — from active to poised to inactive — and contribute to fine-tuning of expression levels (A). At gene bodies, they discriminate between active and inactive conformations. In addition, exons in active genes have higher nucleosome occupancy and thus more histone H3 lysine 36 trimethylation (H3K36me3) and H3K79me2-modified histones than introns (B). (Adapted from [556])

Numerous histone modifying enzyme families, adding and removing covalent groups, have been characterized [558]. Modifications are always reversible and highly dynamic, just like the chromatin structure itself. Many studies have been undertaken to characterize the functions of these vast possibilities of modifications. Some modifications directly influence the interaction of DNA with histones. Acetylation for example neutralizes the negative charge of the lysine residue and phosphorylation of lysine even adds a negative charge. Both effectively reduce the positive charge of histone proteins and thereby the attracting force to the negatively charged DNA strand. Therefore these modifications decompact the chromatin structure. By now, we know that promoters, as well as gene bodies, are characterized by the occurrence of specific chromatin marks, according to their state of activation (Figure 37). Certain marks have been directly associated with transcriptional activation, such as tri-methylation of histone H3 lysine 4 (H3K4me3) on transcriptional start sites, H3K36me3 throughout actively transcribed genes, and H3K4me1 in enhancer regions [559-561]. On the opposite, there are marks associated with repression of transcription, such as H3K9me3, H3K27me3, and H4K12ac [562].

Furthermore, histone marks can act as docking sites for chromatin-associated factors such as remodeling complexes. Many distinct domains have been characterizes for protein-histone docking (Figure 38 A). Acetylated lysine for example can be bound by the bromodomain and methylated lysine by the chromodomain of interaction partners [563,564]. But they can also act in the opposite way, by inhibiting the binding of transcription repressive complexes. For example, H3K4me3 inhibits binding of the NuRD complex [565]. In some cases, cooperation of marks is needed for the effective recruitment of chromatin interacting factors.

Another level of complexity is added by several possibilities of cross talk between histone modifications [566] (Figure 38 B). There can be competitive antagonism, for example in the case of lysine methylation or acetylation, often resulting in opposite outcomes concerning gene transcription. Another possibility is trans-regulation, where one mark obligatory precedes the occurrence of another mark. On the other hand, one mark can also be disruptive for the occurrence of an adjacent modification.

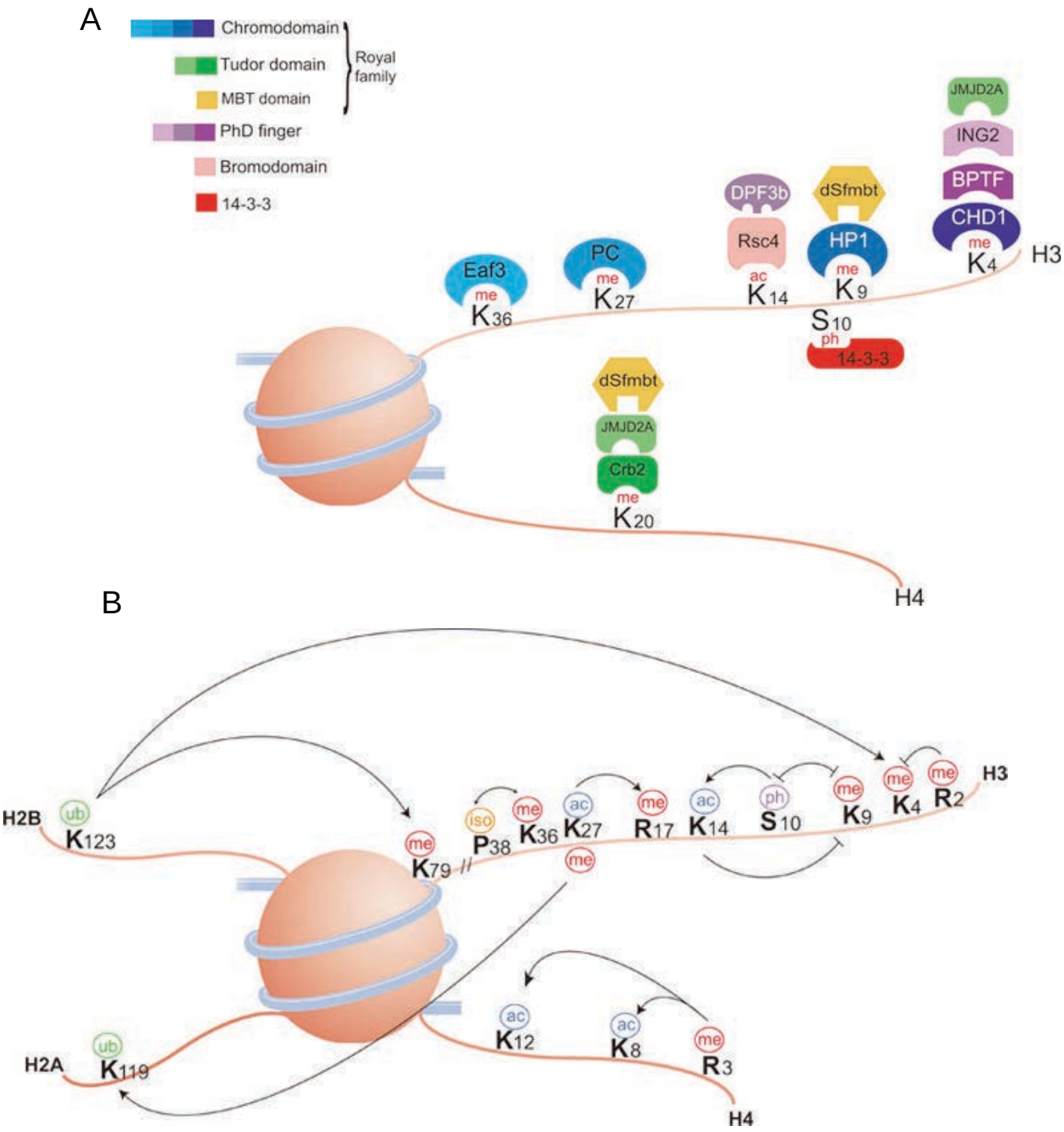


Figure 38: Specific binding of histone marks by protein domains and crosstalk in between marks. Examples of proteins with domains that specifically bind to modified histones as shown (A). Histone modifications can positively or negatively affect other modifications. A positive effect is indicated by an arrowhead and a negative effect is indicated by a flat head (B). [550]

III.2.2 The histone code hypothesis

All these observations bring us to the discussion of the so-called “histone code”. Strahl and Allis first postulated this description of the language of histone marks in the year 2000 [567]. They formulated the hypothesis that different combinations of histone modifications generate a code for downstream regulation and function. As the role of chromatin and chromatin modifications is more and more unraveled their hypothesis is a highly discussed topic [568]. Some of their claims did hold true, as the prediction of histone marks as binding partners for chromatin interacting complexes, while others are still lacking evidence *in vivo* or have even been dismissed.

III.2.2.1 Histone marks as recruiting code

As biochemical approaches identified histone marks, which act as recruiting and binding sites for recognition motifs in chromatin remodeling complexes, the question about a specific code for recruitment remains open (Figure 38 A). Some individual chromatin interacting proteins contain more than one binding domain and the complexity is additionally increased by the formation of protein complexes with multiple histone binding domains. The preferred binding of chromatin regulatory proteins to combinations of histone marks as compared to single marks has been described for multiple examples and suggests a role for combinatorial complexity [568,569]. Still the discovered preferences for binding *in vitro* are often not translatable to observations *in vivo*. Protein complexes are not always found at genomic loci where the predicted specific mark occurs. On the opposite side, they also bind to loci, which lack the specific mark. These observations raised the idea that histone marks could play a rather activating than recruiting role on chromatin regulatory complexes.

III.2.2.2 Histone marks encoding different physiological outcomes

Besides directing the recruitment of chromatin modifying complexes, distinct combinations of marks should predict and allow discrimination between different physiological outcomes. First of all, it has to be said that despite the numerous possible combinations of histone marks mapping studies find only few and simple histone modification patterns *in vivo* [557]. This questions the relevance of combinatorial complexity and the possibility of discrimination of a code leading to specific differential outcomes. Nevertheless, certain marks are associated with a specific physiological process. For example, H3K4me3 together with H3K9ac is

found in the promotor region of actively transcribed promoters across all studied species. But whether their role in these processes is really essential is questionable as a study by Lenstra *et al.* showed. They found that mutation of H3K4 or the K4 methylase had almost no effect on gene expression in yeast [570]. Another study by Martin and colleagues showed that the loss of different acetylation marks on histone 3 is indistinguishable by the yeast cell and moreover shows only modest differences in gene expression [571].

Therefore the histone code *in vivo* is still mainly undeciphered and remains a highly interesting field of study. The main challenge will be to bridge the discrepancy between observed *in vitro* binding of protein complexes to certain combinations of marks and the discrimination and relevant function of mark combinations *in vivo*.

III.3 Regulatory processes of inducible gene expression

Now, that we have understood how the human genome is organized into chromatin and how the components of this structure can be modified and remodeled, we will take a look at the regulatory processes, which allow for inducible gene expression. These processes allows the cell to rapidly react to external stimuli such as stress, infection or developmental cues and to produce new proteins according to the environmental changes. Several genes can be expressed synergistically to produce the suitable response. Nevertheless, there are many levels of selectivity of expression, which tailor the response to a specific stimulus. Mechanisms of regulation can be cell-specific, signal-specific and gene-specific. This chapter will describe the rapid induction of the inflammatory response as an example of inducible gene expression. The inflammatory gene repertoire is activated within minutes upon stimulation. The expression of these genes has to be tightly orchestrated and has to include negative feedback loops in order to prevent tissue damage by ongoing inflammation. Much effort has been made in detailing the sensing, signal transmission and transcriptional and post-transcriptional regulatory mechanisms of the inflammatory response. Transcription factors and chromatin add an additional level of complexity to this regulation. On the transcriptional level, inflammatory response genes can be divided into two classes: primary response genes and secondary response genes. Primary response genes are genes which are rapidly induced and do not require *de novo* protein synthesis [541]. Secondary response

genes, on the other hand, require *de novo* synthesis of transcription or signaling factors and their induction is therefore slower. Differences between these two groups of genes will be discussed as follows. But first of all, the basic processes of general transcription will be described.

III.3.1 Transcription by the general transcription machinery

In the initial step, transcriptional activators bind sequence-specific near the target gene (Figure 39 a). They then recruit large multi-subunit co-activator complexes and nucleosome-remodeling complexes, which facilitate the access to the promotor and the recruitment of the general transcription machinery (Figure 39 b). Multiple enzymes facilitate access by repositioning or ejection of nucleosomes and modifications of histone proteins, as discussed later in this chapter. The pre-initiation complex (PIC) is formed on the promotor by recruited general transcription factors (TF) IIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH and the RNA polymerase II (Pol II) (Figure 39 c). In the next steps, CDK7 in human TFIIH phosphorylates the serine 5 (S5) position of the Pol II carboxy-terminal domain (CTD). At the same time, another subunit of TFIIH, the DNA helicase XPB (xeroderma pigmentosum B), remodels the PIC at the transcription start site to introduce a single-stranded DNA template of 11–15 bases into the active site of Pol II (Figure 39 d). Then, Pol II clears the promotor and transcribes about 20-40 nucleotides into the gene before it is halted at the promotor-proximal-pause site [572]. Now, a second phosphorylation of Pol II at the S2 position of the CTD by CDK9 (Cyclin-dependent kinase 9), a subunit of human P-TEFb (positive transcription elongation factor), is required for continued productive elongation (Figure 39 e). This phosphorylation creates binding sites for chromatin modifying enzymes and nucleosome remodeling complexes, which facilitate the continuing passage of Pol II and the transcription of the full length gene [573].

III.3.2 Activation of poised Pol II

Even though signal-dependent Pol II recruitment and transcription initiation is considered as an important mechanism for induction of gene expression, it has been shown that Pol ll is already poised at many inducible genes in genome-wide analysesin human and murine embryonic stem cells and *Drosophila* cells [575-577].

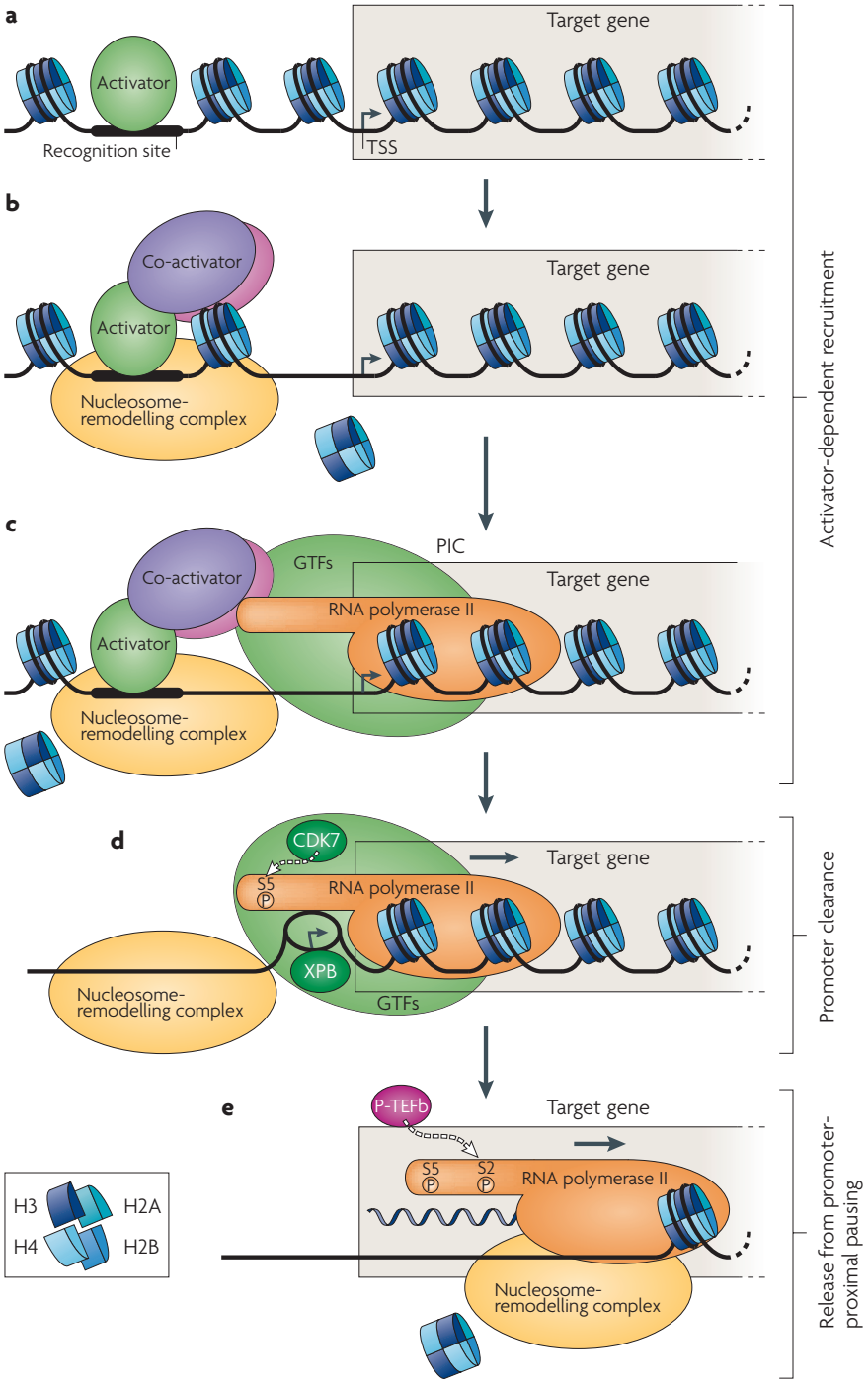


Figure 39: Initiation of transcription by Pol II. Transcription is initiated by sequence specific recruitment of activators (a), subsequent recruitment of co-activator and nucleosome remodeling complexes (b), general transcription factors, and Pol II to the target promoter (c). CDK7 mediated phosphorylation at S5 activates Pol II and XPB mediated remodeling of the PIC leads to introduction of a single-stranded DNA template into the active site of Pol II (d). Pol II clears the promoter and transcribes 20 – 40 nucleotides into the gene until an promoter-proximal pause site. Then, Pol II undergoes a second phosphorylation at the S2 position of the CTD by P-TEFb, which initiates productive elongation (e). [574]

Furthermore, concrete examples have been described, such as the heat-shock genes in *Drosophila melanogaster* and the gene encoding the mammalian transcription factor c-myc [575,578]. In those cases the signal-dependent step is not Pol II recruitment, but the transition from Pol II initiation to Pol II elongation [572]. Hargreaves and colleagues could show that LPS induced acetylation of H4K5, 8 and 12 leads to the recruitment of P-TEFb. This in turn mediates the secondary phosphorylation of paused Pol II and thereby the transition to elongation [579]. It has been suggested that the localization of paused Pol II at inactive inducible genes additionally facilitates the synchronized expression of several genes, which act together in the response to a single stimulus [580].

III.3.3 Differential requirements for chromatin remodeling

The importance of the chromatin structure in the processes of gene expression has become quite clear. A simple difference in kinetics and inducibility of gene expression can be made by the accessibility of the promoter, for example in the case of primary versus secondary response genes. It was found that promoters of primary response genes contain CpG islands, which generally destabilize the assembly of nucleosomes [581,582]. *In vitro* experiments could show that the presence of nucleosomes inhibits formation of the PIC and subsequent transcription [583]. Furthermore, CpG islands contain more binding sites for constitutive transcription factors such as specificity protein 1 (SP1) [581]. These features might allow rapid transcriptional activation without the requirement for chromatin remodeling.

On the other hand, most secondary response genes, but also late primary response genes were found to be dependent on chromatin remodeling induced by LPS stimulation in macrophages, reflected by the differences in NF-κB binding kinetics to different target gene promoters [584]. Also later studies by Ramirez-Carrozzi *et al.* showed a gene variable requirement for the remodeling complex SWI/SNF in the same setting [581]. Recent genome wide studies in yeast found that promoters of inducible genes have a higher density of nucleosomes, highlighting the role of remodeling complexes in gene induction [585].

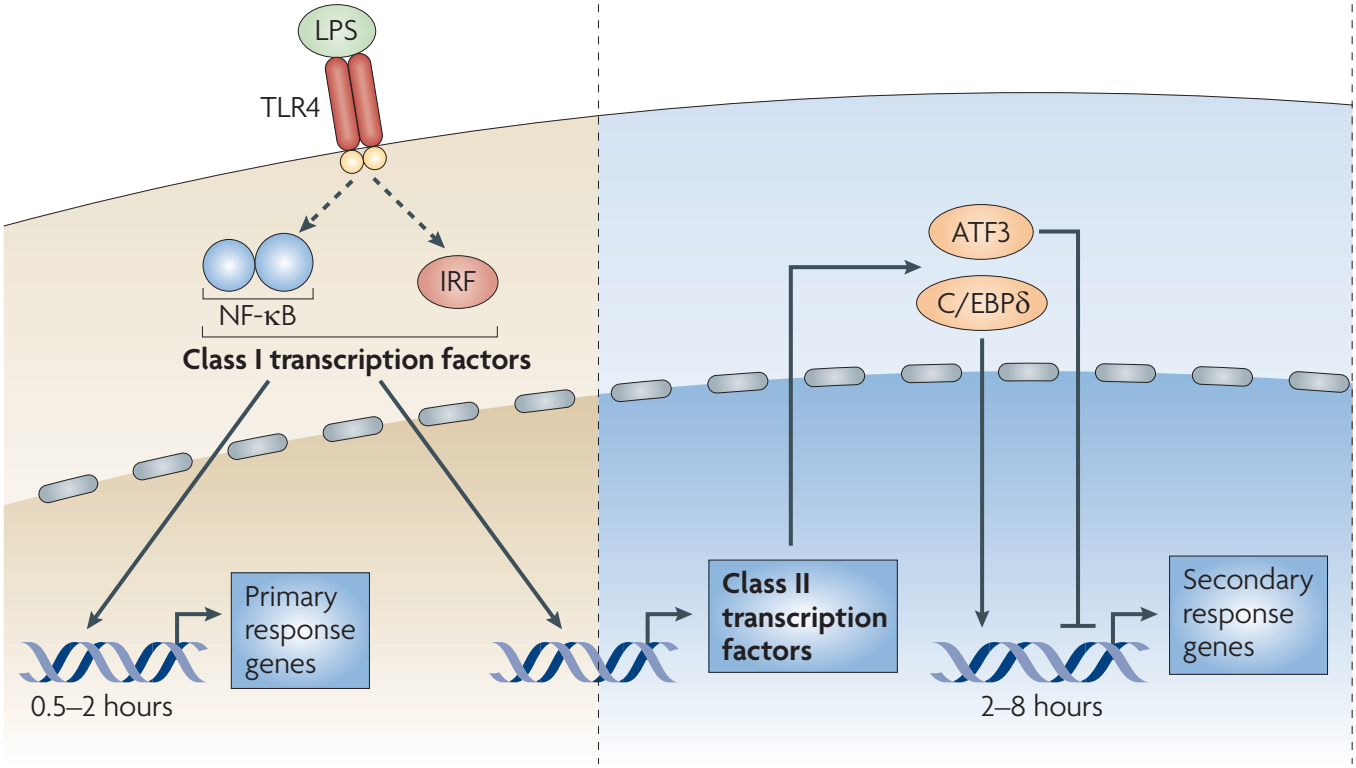


Figure 40: Inflammatory signals induce expression of primary and secondary response genes by different categories of transcription factors. Class I transcription factors are poised in the cytosol and activated post-translationally upon inflammatory signaling. These transcription factors, such as NF-κB or interferon-regulatory factors (IRFs), control the induction of the primary response genes (left panel). Class II transcription factors are induced during the primary response, such as CCAAT/enhancer-binding protein-δ (C/EBPδ), which control induction of the secondary response genes (right panel). [586]

III.3.4 Rapid activation by poised transcription factors

Besides their more accessible promoter structure, another reason for the fast induction of primary response genes is their regulation via transcription factors that are already present in the cell cytosol in a poised state. Most inflammatory transcription factors are constitutively expressed and activated by signal-dependent post-translational modifications, commonly phosphorylation or dephosphorylation of the factors themselves or of inhibitory proteins. Usually these transcription factors are retained in the cytoplasm in the basal state and translocate to the nucleus upon signal-dependent activation. The first transcription factor discovered to directly induce primary response genes was NF-κB. LPS was shown to stimulate the post-transcriptional activation of cytosolic NF-κB, followed by sequence-specific DNA binding activity and gene transcription [587] (Figure 40, left panel). Multiple transcription factors, which are induced by inflammatory stimuli and activated via post-translational modifications have been discovered thereafter: for example AP1 [588], cyclic-AMP response element-binding protein (CREB) [589], E2F [590], and NFAT (Nuclear factor of activated T-cells) [591]. It should be mentioned that this first round of transcription factors induces the expression and *de novo* synthesis of a second round of transcription factors involved in the expression of secondary response genes (Figure 40, right panel). Thereby, the inflammatory response can be adapted in a second level of regulation [586].

III.3.5 Enhancer motifs

As mentioned above, transcription factors bind to specific DNA motifs located at the transcription start site in the gene promotor. This is one way to promote specificity in gene activation. Another way is via the involvement of distant enhancer motifs [592]. These DNA motifs can be located upstream or downstream of the gene or even on a different chromosome. By looping of DNA, they can come into close contact with the gene of interest and can help to deliver transcription factors to their binding sites in the promotor region (Figure 41). Enhancer motifs bind enhancer proteins, which help to recruit Pol II and the general transcription machinery. Whole complexes of multiple inducible transcription factors, which activate transcription synergistically have been described, such as the so-called enhanceosome [593].

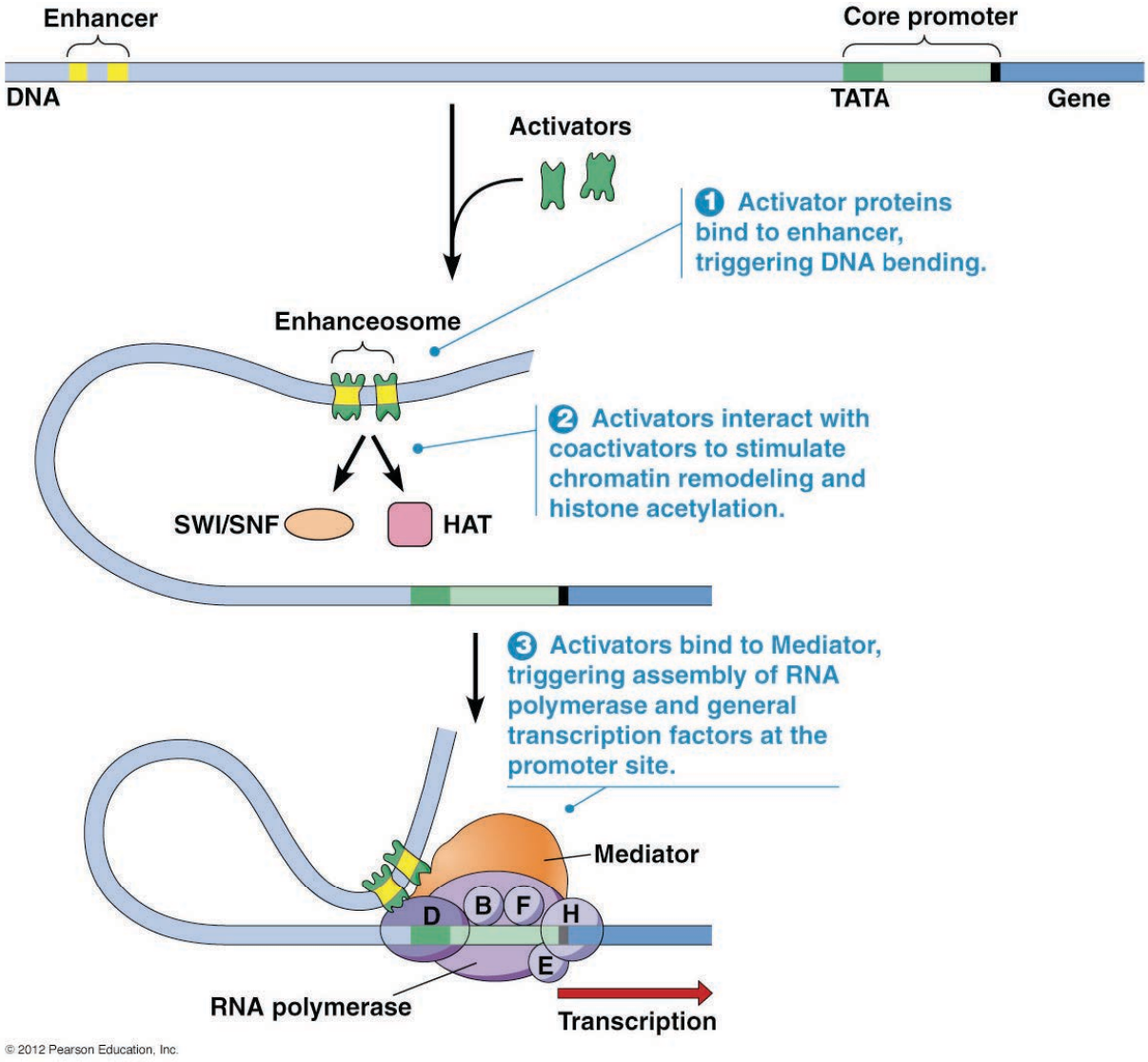


Figure 41: Action of distant enhancers on gene expression. Enhancers can be located at a great distance from their target gene. Regulatory transcription factors (activators) bind to the enhancer sequence and form the enhanceosome (1). They induce looping of DNA, which brings the enhancer close to the target gene promoter (2). Furthermore, they interact with specific coactivators and chromatin remodeling complexes, to transform the chromatin environment for the recruitment of the general transcription factors and Pol II to the promoter by the multiprotein complex Mediator, which is essential for activator-dependent transcription (3). (Pearson Education Inc.)

The enhanceosome was described in the activation of the interferon β gene, an important protein for fighting viral infection. It consists of several different DNA-binding proteins, such as ATF2 (Activating transcription factor 2)/c-Jun, interferon response factors (IRFs) and NF-κB. This protein complex recruits histone acetyltransferases (HATs) and remodeling complexes leading to nucleosome replacement and recruitment of the general transcription machinery [594]. Gene expression is only activated if the stimulus activates all transcription factors associated with the enhanceosome [595]. This example emphasizes the importance for synergy in the specificity of the transcriptional outcome. It can be speculated that such a “transcription factor code” could also direct specificity for other inducible genes.

III.3.6 DNA methylation

Methylation of DNA on cytosines within CpG dinucleotides is another level of regulation of gene expression [596]. DNA methylation in mammals is achieved by three distinct phylogenic DNA methyltransferases (DNMT) 1, 3a and 3b [597,598]. DNA methylation patterns show a tight correlation with chromatin structure, as active regions of the chromatin are associated with hypomethylated DNA, whereas chromatin with hypermethylated DNA is inactive [597]. Recent evidence has shown that there is a connection between DNA methylation and histone modifications and that specific DNA methylation patterns could be directed by chromatin modification. Several histone modifying enzymes, such as the histone deacetylases (HDAC) 1 and HDAC2, interact with DNA methylating enzymes, such as DNMT1 and 3a, and recruit them to specific targets [599]. Thereby, the repressive abilities of histone methylation and DNA methylation can be coordinated at target genes [600]. Furthermore, some histone interacting proteins show preferences for binding to chromatin associated with methylated or non-methylated DNA [601]. There has been a report for enhanced binding of chromatin interacting protein Ubiquitin-like, containing PHD and RING finger domains 1 (UHRF1) to nucleosomes with methylated DNA. An opposing observation has been made for the lysine-specific demethylase 2A (KDM2A), which only binds to H3K9me3 when the connected DNA is not methylated.

III.3.7 Removal of repressive histone marks and complexes

As mentioned earlier, several studies have identified the presence of repressive histone marks H3K9-me3, H3K27-me3 and H2AK119-ub at the promoters of inducible genes [602-604] (Figure 39). These marks were rapidly lost upon LPS stimulation in macrophages. Removal of repressive marks upon stimulation required the recruitment of demethylases and deubiquitinase to target genes. Furthermore, repressive protein complexes, such as NCoR (nuclear receptor corepressor) and SMRT (silencing mediator of retinoic acid and thyroid hormone receptors), have been found associated with primary response genes [605,606]. These repressor complexes are often associated with HDACs, which additionally help to maintain a silenced chromatin state. Removing such repressing marks and complexes presents another level of gene activation upon stimulation.

III.3.8 Activating histone marks

Besides, repressing gene transcription, histone marks can also work in the opposite way. The promoters of primary response genes show a high level basic of transcription activating histone marks, such as H3K4me3 and H3ac [579,607] (Figure 39). Promoters of secondary response genes on the other hand only subsequently acquire these marks upon LPS stimulation.

III.3.9 Non-coding regulatory RNAs

There is emerging evidence for the delivery and control of histone marks by non-coding regulatory RNAs, which have been shown to interact with and direct the actions of histone modifying enzymes [608,609]. Recent studies have discovered the huge abundance of large multi-exonic intervening non-coding RNAs (lincRNAs). Their sequences are highly evolutionary conserved speaking for their functionality. Guttman *et al.* characterized their association with functional gene sets of immune response, response to pathogens, biotic stimuli, cell surface receptor, and transmembrane signaling [610]. Moreover, they have been identified as players in the regulation of gene expression. LincRNAs were found located near genes encoding transcription factors, and other neighboring genes were strongly biased towards those encoded transcription factors [610]. The two lincRNAs homeobox antisense intergenic RNA (HOTAIR) and X-inactive specific transcript (XIST) were found associated with chromatin remodeling complexes and were shown to represses gene

expression [611,612]. On the other hand, Lefevre *et al.* could show the activating activity of the noncoding RNA LINoCR (LPS-inducible non-coding RNA) on transcription of the chicken lysozyme gene upon stimulation with LPS. Expression of LINoCR is correlated with IκB kinase (IKK) recruitment, the subsequent phosphorylation of histone H3 at serine 10 (H3S10-P) together with H3K9-ac in the transcribed region and the eviction of the enhancer-blocking protein CTCF [613].

III.3.10 Activator complexes

A special role in activation of gene expression is played by transcriptional co-regulators, which lack DNA-binding specificity and are recruited to target genes through protein-protein interaction or interaction with histone marks (Figure 40 A). They possess histone-modifying and chromatin-remodeling activities and thereby aid transcription factors in the induction of gene expression. The transcription factor NF-κB for example cooperates with its co-activators CBP/p300 (CREB-binding protein/E1A-associated protein of 300kDa). They execute HAT activity and recruit the remodeling complex SWI/SNF and other factors [614]. Two other histone acetyltransferases, PCAF (p300-CBP-associated factor) and GCN5 (general control non-repressive 5) were shown to direct transcription elongation factors to target genes [579]. The role of these complexes will be discussed in greater detail in chapter VI. Of special interest for gene activation is the role of histone acetylation by HAT complexes, as will be discussed in the following chapter.

III.4 What we know about regulation of AMPs gene expression

As we have discussed in chapter II.7, multiple and various internal and external stimuli are involved in the induction and repression of AMPs expression in the human body. If we consider the above-discussed mechanism, which govern inducible gene expression, we must say that relatively little is known of their influence in the expression of AMPs. This section summarizes the knowledge we have concerning the activation of AMPs expression by stimulus-induced transcription factors, in mammals as well as in *Drosophila*. The second part will give a short outlook on the involvement of epigenetic regulatory mechanism.

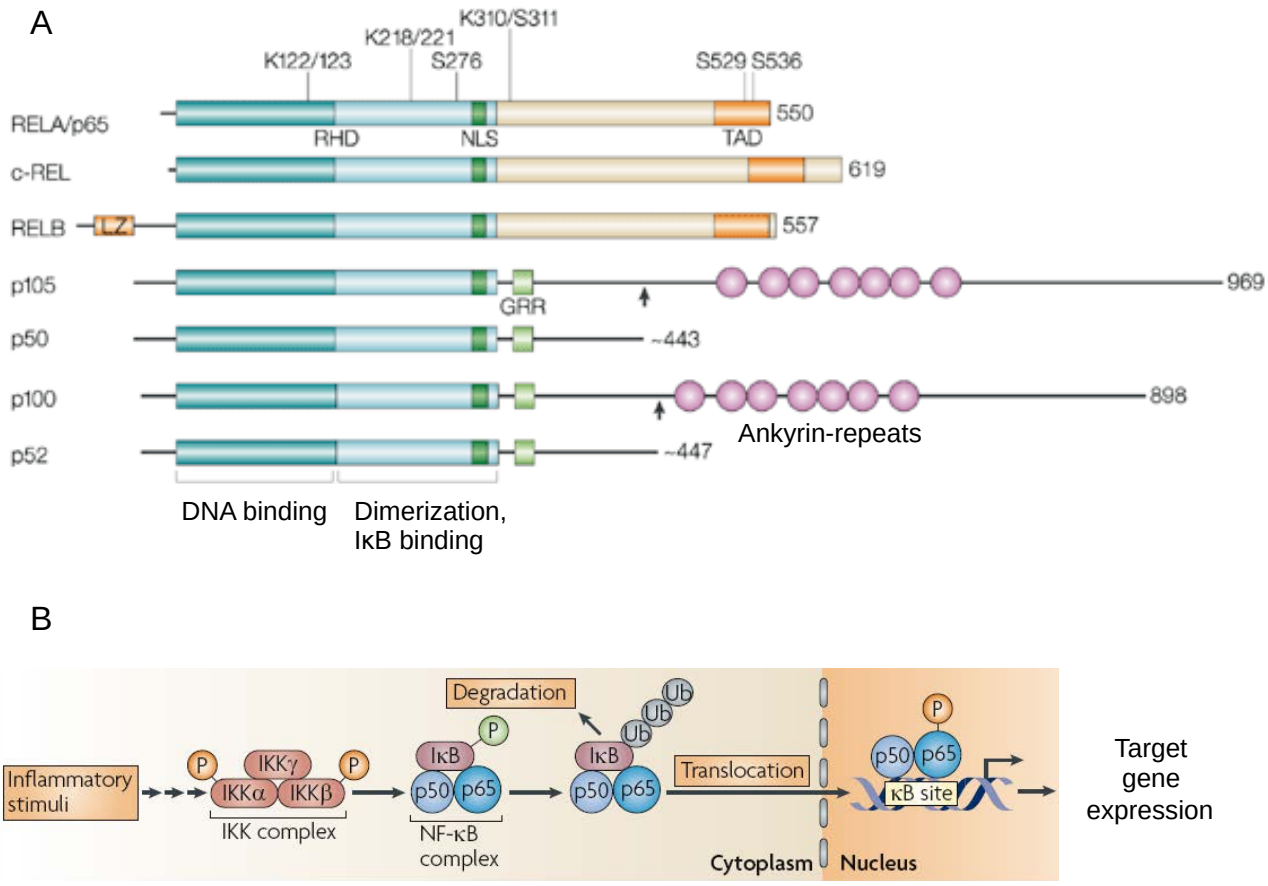


Figure 42: The NF-κB family members and activation of the NF-κB pathway. The mammalian NF-κB/Rel family of transcription factors comprises 5 members: RelA/p65 (RelA), RelB, c-Rel, NF-κB1 (p105 cleaved to p50) and NF-κB2 (p100 cleaved to p52) (**A**). They all contain REL-homology domains (RHDs), which mediate DNA-binding, dimerization and binding of the inhibitors IκB. Only p65, c-REL and RelB contain carboxy-terminal transactivation domains (TADs) and possess transcriptional activity. The p105 and p100 proteins contain ankyrin-repeats as well as glycine-rich regions (GRRs). Phosphorylation of p65 at serines S276, S311, S529 and/or S536 is required for optimal transcriptional activity. Acetylation of p65 at lysines K122, K123, K218, K221 and K310 regulates distinct functions of NF-κB, including DNA binding, and IκB association. The leucine zipper (LZ) of RelB is required for transactivation by RelB. In the simplest model of NF-κB activation, an inflammatory stimulus activates signal transduction pathways that induce the activation of the IKK complex (**B**). This results in the phosphorylation of IκB proteins and consequently their degradation by the proteasome. Released NF-κB dimers translocate to the nucleus and bind κB sites in the promoters or enhancers of target genes, which leads to their transcription. Green phosphate: negative regulation of the phosphorylated protein; Yellow phosphate: indicates activation of the phosphorylated protein; Ub, ubiquitin. (Adapted from [618] and [619])

III.4.1 Transcription factors involved in the expression of AMPs

It has already been mentioned that the expression of AMPs is mediated via inflammatory receptors, such as TLRs, and signaling pathways, such as the MAPK pathway. Most of these pathways converge in the activation of transcription factors such as NF-κB or AP1. Interestingly, not only inflammatory pathways, but also situations related to stress and nutrition activate AMP expression and some transcription factors acting in those situations have been described, such as HIF1 and the *Drosophila* transcription factors FOXO and caudal. The following chapter summarizes the knowledge about transcription factors acting on AMP promoters.

III.4.1.1 NF-κB

The transcription factor NF-κB can be activated by a range of stimuli, including various pro-inflammatory cytokines, growth factors, DNA-damaging agents and MAMPs. Its role in transcriptional activation is versatile as it is involved in multiple cellular processes, such as inflammatory and immune responses, apoptosis, cell proliferation and differentiation, and tumorigenesis [615,616].

III.4.1.1.1 The NF-κB family

There are five members belonging to the nuclear factor kappa B transcription factor family: p65 (RelA), RelB, c-Rel, NF-κB1 (p105 cleaved to p50) and NF-κB2 (p100 cleaved to p52) [617] (Figure 42 A). They all contain an aminoterminal Rel homology domain (RHD), which is essential for DNA binding, dimerization and interaction with the IκB family of NF-κB inhibitors. Additionally, RelA, c-Rel and RelB contain transactivation domains (TADs) within the carboxy-teminal region, which facilitate interaction with various components of the basal transcription apparatus and co-activators. The active transcription factors are formed by homo- or heterodimers of different family members, whereby the typical complex consists of p50 and p65. Combinations of p50/p65, p50/c-Rel, p65/p65, and p65/c-Rel are all transcriptionally active, whereas p50 or p52 homodimers are transcriptionally repressive.

II.4.1.1.2 Activation of NF-κB

In unstimulated cells, NF-κB is kept in the cytosol by binding to a complex of members of the IκB family of cytoplasmic inhibitors, which prevent translocation into the nucleus [620] (Figure 42 B). Upon stimulation, the IκB-kinase (IKK) complex is

activated and mediates site-specific phosphorylation of the IκBs, which is followed by their ubiquitination and proteasomal degradation. NF-κB is released and translocates into the nucleus, where is recruits co-activators and binds to target gene consensus sequences for gene transcription [621]. Additionally, its activity and target-gene specificity can be directed by post-translational modification, such as phosphorylation and acetylation and modification of the target gene surrounding chromatin environment [619] (Figure 42 A). These characteristics make it an interesting target in the context of epigenetic/post-translational regulation. Finally, it induces the transcription of its own repressor IκBα, which mediates shuttling out of the nucleus and terminates the transcriptional response in a way of negative feedback [622,623].

III.4.1.1.3 The role of NF-κB in DEFB2 expression

Several studies showed that induction of DEFB2 expression in IECs upon bacterial challenge, is dependent on NF-κB. This was observed for example upon infection with enteroinvasive *Salmonella enterica* serovar Dublin or enteroinvasive *E. coli* [280], stimulation with *S*. *enterica* serovar Enteritidis flagellum filament protein [528], or stimulation by LPS [530].

Furthermore, infection of gastric cells with *H. pylori* resulted in NF-κB-dependent induction of DEFB2 [624]. Also, the stimulation of DEFB2 expression by cytokines was shown to be mediated by NF-κB. For example IL1β induced expression in lung epithelial cells A549 or corneal epithelial cells [502,625]. A study in human airway epithelium showed the importance of the NF-κB binding sites in the DEFB2 promotor for the expression upon LTA stimulation [532].

The promoter region of the DEFB2 gene contains several binding sites for NF-κB [286,626] (Figure 51). Wehkamp *et al.* investigated the importance of the two binding sites (at positions -205 to -186 and -596 to -572) for the expression of DEFB2 in IECs upon infection with *E. coli* Nissle 1917 via a site-directed mutagenesis approach [627]. They found that mutation of the first proximal binding site almost completely abolished the induction of the DEFB2 promoter, whereas mutation of the second potential binding site showed no significant effect. Additionally, they used the specific NF-κB inhibitor helenalin, which blocked *E. coli* Nissle 1917-mediated DEFB2 induction. Two other studies also identified these two NF-κB sites as essential for LPS-dependent DEFB2 induction in macrophages and HeLa cells [628,629].

III.4.1.2 AP1

Another important example for cellular signaling induced by various stimuli is the activation of the MAPK phosphorylation cascades, for example by stress, inflammation or growth factors (Figure 43). In mammals, 4 MAPK cascades have been described, consisting of different subtypes and responding to different stimuli: the extracellular signal-regulated kinase (ERK 1 and ERK 2) pathways, the JNK (JNK1, JNK2 and JNK3) pathway, the p38 (p38α, p38β, p38δ and p38γ) pathway and the big mitogen-activated protein kinase (BMK1/ERK5) pathway [630]. Downstream of subsequent phosphorylation events, activation of the MAP kinase pathways results in the phosphorylation of transcription factors and coregulators bound to their regulatory elements in the promoters of target genes. One of them is AP1, which is a homo- or heterodimer of proteins belonging to the c-Fos, c-Jun, ATF and JDP (Jun dimerization protein) families [631].

The promotor region of DEFB2 contains several AP1 binding sites [286,626]. Several studies have described the involvement of MAPK pathways and of AP1 in the expression of DEFB2 by the use of specific inhibitors of those pathways or site-directed mutagenesis of the promotor region. Interestingly, the results are not always the same. A study in IECs by Wehkamp *et al.* showed the involvement of the JNK pathway (but not p38 or ERK1/2 pathways) and AP1 in DEFB2 expression upon infection with the probiotic *E. coli* Nissle 1917 [627]. Another study in IECs showed the involvement of JNK pathway and AP1 in LPS-TLR4 mediated up-regulation of DEFB2 [530]. On the contrary, Ogushi *et al.* showed that *Salmonella* FliC stimulation in IECs increased phosphorylation of p38 and ERK1/2 (but not JNK) and results in AP1-mediated transcription of DEFB2 [632].

In other cell types, similar results for the involvement of different MAPK pathways were obtained. Krisanaprakornkit *et al.* showed that *Fusobacterium nucleatum* induces DEFB2 in human gingival epithelial cells via the p38 and JNK kinase pathways [634]. A study in the lung epithelial cell line A549 demonstrated the involvement of p38, JNK (but not ERK1/2) in the induction of DEFB2 expression upon IL1β treatment [625]. The same findings were obtained in a study in corneal epithelial cells [502]. IL1α on the other hand was found to upregulates DEFB2

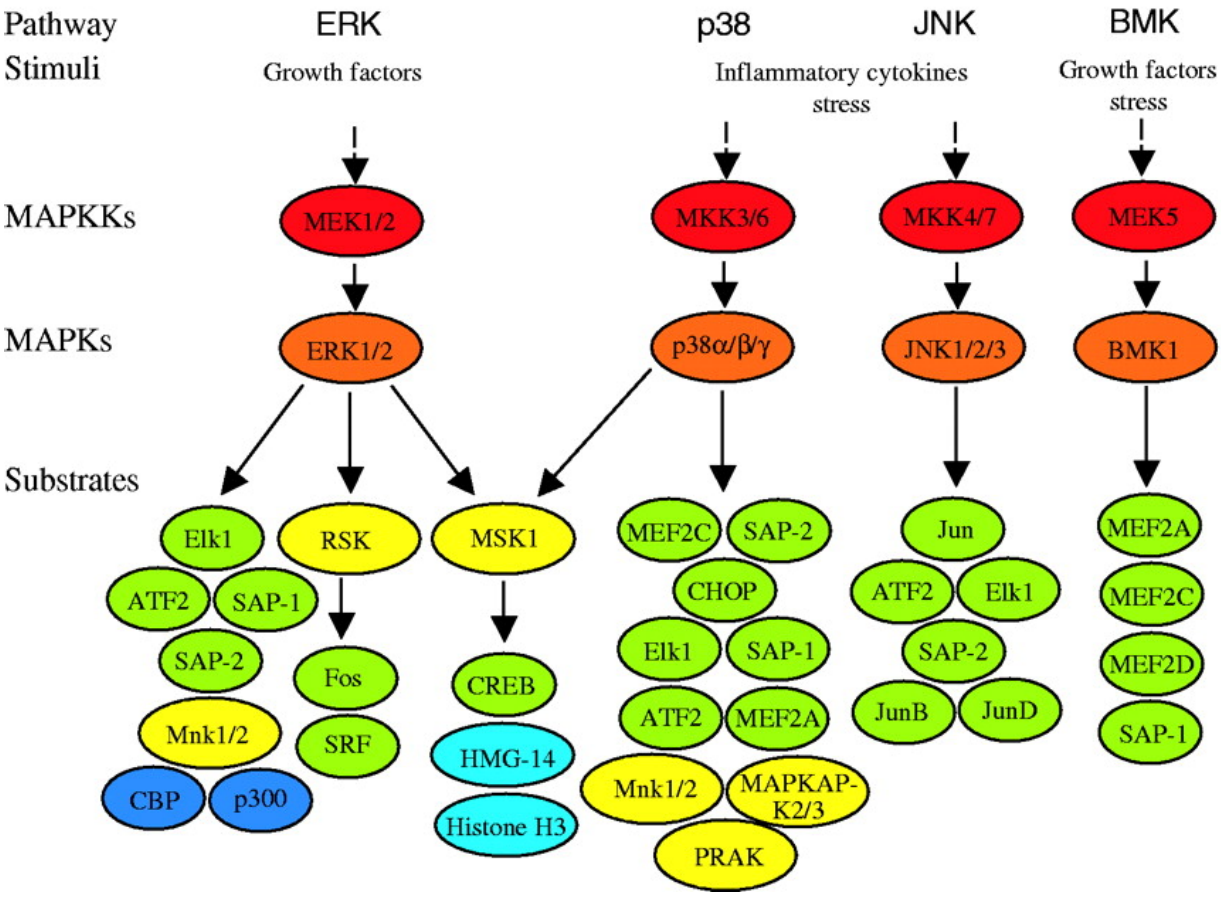


Figure 43: Mammalian MAPK pathways. Schematic overview of the mammalian mitogen-activated protein kinase (MAPK) pathways and some of the downstream substrates. Color code as follows: red, MAPKKs; orange, MAPKs; yellow, downstream kinases; green, transcription factors; light blue, nucleosomal proteins; dark blue, coregulators [633].

transcription via the activation of the ERK1/2 signaling pathway in human middle ear epithelial cells [486].

III.4.1.3 Hypoxia inducible factor 1 (HIF1)

The environment in the intestine is characterized by a steep oxygen gradient from the anaerobic lumen across the epithelium into the highly vascularized sub-epithelium. IECs are uniquely resistant to this ‘‘physiological hypoxia’’ and have developed measures to cope with this challenging situation to maintain their absorptive and barrier properties [635]. The main responder to changing oxygen conditions and hypoxia is the transcription factor HIF1, which is a heterodimer of an α- and a β-subunit [636]. HIF1α is an important regulator of intestinal homeostasis as it is responsible for example for the expression of mucin-3, and the intestinal trefoil factors [637,638]. A recent study by Kelly *et al.* established a link between HIF1α and AMPs expression in the intestine [639]. They found that mice with a targeted intestinal epithelial HIF1α knockout showed decreased expression of a cluster of 11 defensin-related genes. Interestingly, studies in these mice identified HIF1α as a protective factor of experimental colitis [640]. Knockdown of both HIF1 subunits in two human intestinal epithelial cell lines revealed the regulation of DEFB1 (but not DEFB2 or 3) by HIF1α via binding to the a single HIF1 responsive element (HRE) in the promotor region. This study indicates a role for HIF1α in the constitutive expression of DEFB1 and thereby the maintenance of homeostasis in the hypoxic environment of the intestine.

III.4.1.4 Forkhead box transcription factor (FOXO)

The transpiration factor FOXO is responsible for the expression of genes involved in cell growth, proliferation and differentiation and has a pivotal role in adapting metabolism to nutrient conditions [641]. An interesting study by Becker *et al.* deciphered a new cross-regulatory mechanism between metabolism and innate immunity in *Drosophila*. Here, AMP expression upon infection is regulated by two distinct signaling cascades, the Toll and IMD pathways, as described in chapter II.1.1. Becker *et al.* found that mutants of the insulin/insulin-like growth factor signaling (ILS) cascade showed an upregulation of AMPs in epithelia and the fat body in non-infected conditions. The same effect was observed by starvation in tissue culture cells, larvae and adult flies. FOXO is the signal transducer of the ILS cascade and is

activated when energy levels are low and ILS is reduced. Sequence analysis of the regulatory regions identified highly conserved FOXO/forkhead consensus binding sites in most drosophila AMP genes, especially in the drosomycin promoter, which contains five putative FOXO binding sites. Moreover, electrophoretic mobility band shift and supershift assays showed that FOXO binds directly to the FOXO sites in the drosomycin promoter. Knockout of FOXO verified its role in the induction of AMPs genes in the above-described conditions. Furthermore, they could show that the regulation is independent of the pathogen-responsive innate immunity pathways by the use of mutants, which render those pathways unresponsive. As FOXO is evolutionarily conserved across species it was interesting to see that regulation of defensins in several human cell lines, such as lung, gut, kidney and skin cells, is also ILS-dependent. This indicates that the FOXO-dependent regulation of AMPs genes is conserved and may help maintaining and strengthening the defense barrier, in particular when animals are suffering from energy shortage or stress.

III.4.1.5 Caudal

In *Drosophila*, similar to the human body, the first line of defense consists of the local expression of AMPs in barrier epithelia, which are in direct contact with microorganisms. This local AMP expression can also either be constitutive or inducible in a tissue-specific manner. κB sites and Rel family transcription factors (Dif and Relish) are essential for inducible expression of all AMPs during systemic and inducible local immune responses [219]. On the other hand, a study by Ruy *et al.* described the involvement of the transcription factor caudal, which is known for its function in anteroposterior body axis formation, in the constitutive expression of a subset of AMP genes in *Drosophila* epithelia, such as the salivary glands and the ejaculatory duct, via the binding to caudal responsive elements (CDRE) in their promoter region [642]. The role of this transcription factor is highly tissue specific, as a later study by the same group identified caudal as a repressor of commensal-mediated NF-κB-inducible AMP expression in the fly gut epithelium, and thereby regulator of gut microbiota composition and homeostasis [643]. Decrease of caudal expression via siRNA knockdown led to overexpression of AMPs, which in turn altered the commensal population within the intestine and finally led to gut cell apoptosis and mortality.

III.4.2 Epigenetic mechanism involved in AMPs expression

The most prominent connection between epigenetic regulatory mechanism and AMPs gene expression that has been made concerns HDAC enzymes. HDACs are important regulators of inflammation and immunity [644]. Therefore it is not surprising that they are involved as well in the regulation of AMP expression. Still, only very little has been described in this regard. The first reports about the involvement of HDACs in AMPs expression were concerning natural HDAC inhibitors. The bacteria produced short chain fatty acid butyrate is such an inhibitor, and was found to induce the expression of LL37 in IECs *in vitro* and in the rabbit colon [264,534]. In a follow up study Schauber and colleagues compared the effects of butyrate to the HDAC inhibitor TSA and found that they both induce expression of LL37 in colonic, gastric and hepatocellular carcinoma cells [645]. Furthermore, treatment with the butyrate and another dietary HDACi, sulforaphane, stimulated DEFB2 expression in colonic epithelial cells [538]. These data are moreover especially relevant concerning the intestine, as it is naturally exposed to HDAC inhibitors coming from the diet and produced by the microbiota.

One recent study was able to single out HDAC1 as the sole HDAC repsonsible for the control of DEFB1 expression in the lung. They found a 2-fold increase in DEFB1 expression after treatment of lung epithelial cell lines with the HDAC inhibitor TSA and in a follow up by knockdown of HDAC1 [646]. ChIP experiments could show an increase in acetylation of H3 and the transcription-promoting histone mark H3K4-me3 at the DEFB1 promoter upon specific HDAC1 inhibitor treatment, highlighting the epigenetic component in the regulation of the DEFB1 expression in the lung.

Collectively these results give us a hint on the involvement of enzymes mediating protein/histone acetylation and deacetylation in the expression of AMPs.

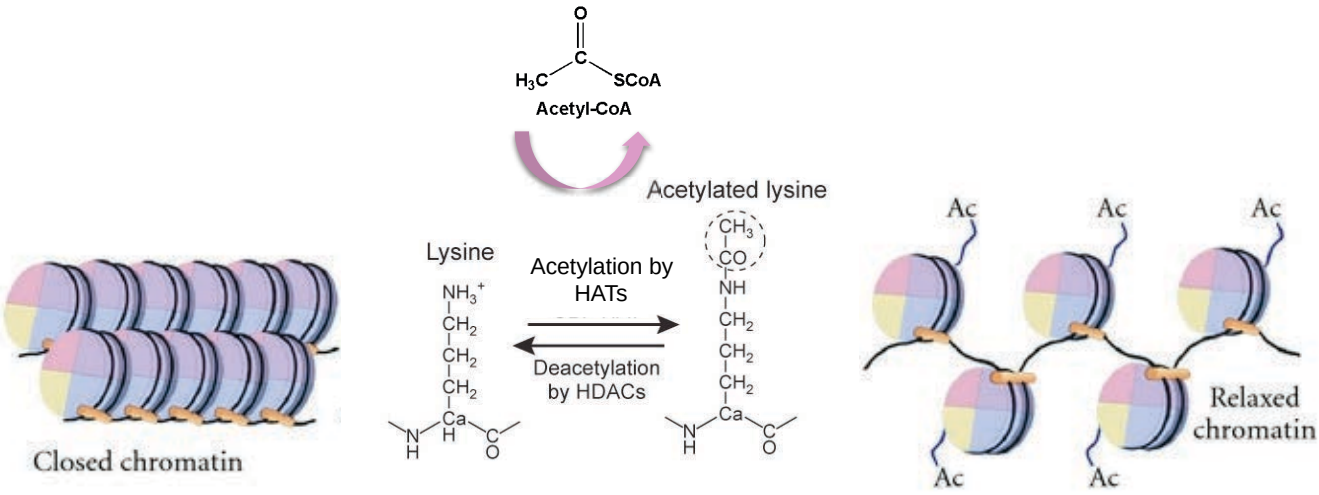


Figure 44: Acetylation and deacetylation of histones by HAT and HDAC enzymes. Histone acetylation is mediated by the addition of an acetyl-group from acetyl-CoA on lysine resides by HAT enzymes. Addition of acetyl groups results in a more open, transcriptionally permissive chromatin conformation (right). Removal of acetyl groups is mediated by HDACs and leads to a condensed, transcriptionally repressive chromatin conformation (left). (Adapted from [648] and [649])

III.5 HAT and HDAC enzymes regulate acetylation of (histone) proteins

In scope of the presented thesis, the focus in epigenetic regulation is laid on the families of HAT and HDAC enzymes and their action on histone and non-histone proteins. The acetylation of histones was discovered in the 60s [647]. Since then, it has become one of the most studied post-translational modification in histones and is broadly associated with activation of gene expression. Two opposing enzyme families regulate acetylation and deacetylation were identified almost 30 years later: the HATs and the HDACs [650] (Figure 44).

III.5.1 HAT enzymes

HAT enzymes transferred acetyl groups from acetyl-coenzym A to the ε-amino group of lysine side chains (Figure 44). They can be classified into two groups: type-A and type-B. The type-A HATs can be categorized into at least three separate families: GCN5-related *N*-acetyltransferases (GNATs), MYST (named after its four founding members MOZ Ybf2 (Sas3), Sas2, and Tip60) and CBP/p300 families [650]. Type-B HATs are predominantly cytoplasmic and acetylate newly synthesized free histones for their deposition into the chromatin structure [651]. In humans there are two GCN5-like proteins, PCAF and GCN5 and five members of the MYST family, MOZ (monocytic leukemia zinc finger protein), MORF (MOZ-related factor), HBO1 (HAT bound to Orc1), Tip60 (Tat-interacting protein of 60 kDa), and MOF (males absent on the first) and p300/CBP.

III.5.2 HDAC enzymes

HDAC enzymes are commonly regarded as repressors of gene transcription, as they remove neutralizing acetyl groups and restore the tight binding of DNA to the chromatin structure (Figure 44). 18 classical HDAC enzymes have been described, categorized into 4 classes. All classical HDACs require Zn^+ for deacetylase activity. Class I HDACs (HDAC1, 2, 3 and 8) are generally localized to the nucleus and, with the exception of HDAC3, lack a nuclear export signal [652]. They have been most widely studied as histone modifiers and transcriptional repressors. The class II enzymes are subdivided into class IIa (HDAC4, 5, 7 and 9) and IIb (HDAC6 and 10) [653]. IIa HDACs possess N-terminal domains that interact with transcription factors.

They also possess C-terminal nuclear export signals, which enable shuttling between the nucleus and cytoplasm. These enzymes primarily control gene expression by recruiting corepressors or coactivators. Class IIb HDACs are distinguished from the class IIa subfamily in possessing tandem deacetylase domains, although the second domain of HDAC10 is reported to be nonfunctional [654]. HDAC6 is unique amongst the classical HDAC family in that it is predominantly cytoplasmic, whereas HDAC10 is found in both the nucleus and cytoplasm. The class III HDACs, also called sirtuins (silent information regulator 2-related proteins), are comprised of seven members (SIRT1-7) and require NAD^+ as cofactor for their activity [655]. Class IV only has one member, the HDAC 11.

III.5.3 HATs and HDACs in multi-protein complexes

HAT and HDAC enzymes are often found in multi-protein complexes together with other histone modifying enzymes. The SAGA (Spt-Ada-Gcn5 acetyltransferase) complex is a well studied example, which is conserved between yeast and humans, and contains enzymatic modules for acetylation and deubiquitination [656]. HDACs on the other hand are found in complexes with demethylases, for example HDAC1/2 is part of multiple different complexes in cooperation with histone demethylases [657]. Furthermore, non-catalytic subunits in a complex can help to target the catalytic subunits to a genomic region and influence their substrate specificity [658]. HAT complexes for example have been found to use methylated histones as docking sites for their histone acetylation activity [659]. Besides, many HATs themselves contain bromodomains for acetyl-lysine recognition [563].

III.5.4 Non-histone targets of HATs and HDACs

Both these enzyme families don't possess great substrate specificity and no consensus motifs have been described so far. It is not surprising that by now many non-histone targets for acetylation and deacetylation by these enzymes have been identified [660,661]. Among those targets are transcription factors, cytoskeletal proteins, molecular chaperones, nuclear import factors and even viral proteins. One interesting non-histone target of HATs and HDACs is the transcription factor NF-κB, whose transcriptional activity is widely regulated by acetylation of lysine residues [662]. The role of acetylation in NF-κB activity will be discussed in more detail in chapter VI. Acetylation of proteins can have various effects, such as stimulation or disruption of

DNA binding, regulation of protein-protein interaction or promotion of protein stability [663]. The fact that different HATs and HDACs have the same target makes their functional studies additionally difficult. The cell cycle regulator p53 for example was found to be acetylated by p300, PCAF and two members of the MYST family respectively [664].

III.6 Epigenetic drugs – inhibitors of histone-modifying enzymes

Epigenetic regulation of gene expression has been related to specific disease states, such as for example cancer, neurological disorders and autoimmune diseases [665]. Therefore the possibilities of treatment of certain diseases with epigenetic drugs is being highly investigated [666]. Whole libraries of natural and synthetic drugs, which target a variety of histone modifying enzymes are being characterized and some of these drugs are tested already in clinical trials (Figure 45). The following chapter will give a brief overview of the epigenetic drugs related to the presented work.

III.5.1 Inhibitors of DNA methylation

Methylation of DNA can be inhibited by selective drugs, for example by 5-azacytidine, which is a catalytic inhibitor of DNMT1 [667]. It incorporates into DNA and traps DNMT1 in the progressing fork, resulting in passive demethylation of the nascent DNA strand [668]. This approach is especially studied in the field of cancer research as it is proposed that inhibition of DNMT1 activates aberrantly methylated tumor suppressor genes and thereby arrests tumor growth [669,670].

III.5.2 Inhibitors of histone methylation and demethylation

As mentioned earlier, histone methylation can have activating or suppressing effects. Inhibitors of histone methyltransferases (HMTs) could prevent silencing of tumor suppressor genes by methylation of H3K9 and H3K27 [672-675] (Figure 45). On the other hand, histone demethylases (HDMs), such as the lysine-specific demethylase 1 (LSD1), are also interesting targets [676]. The nonselective monoamine oxidase inhibitor pargyline for example was found to inhibit LSD1 [677,678] (Figure 45). LSD1 demethylates H3K4Me2, which is a hallmark of active genes. Inhibition of histone demethylation could result in the activation of potential tumor suppressor genes.

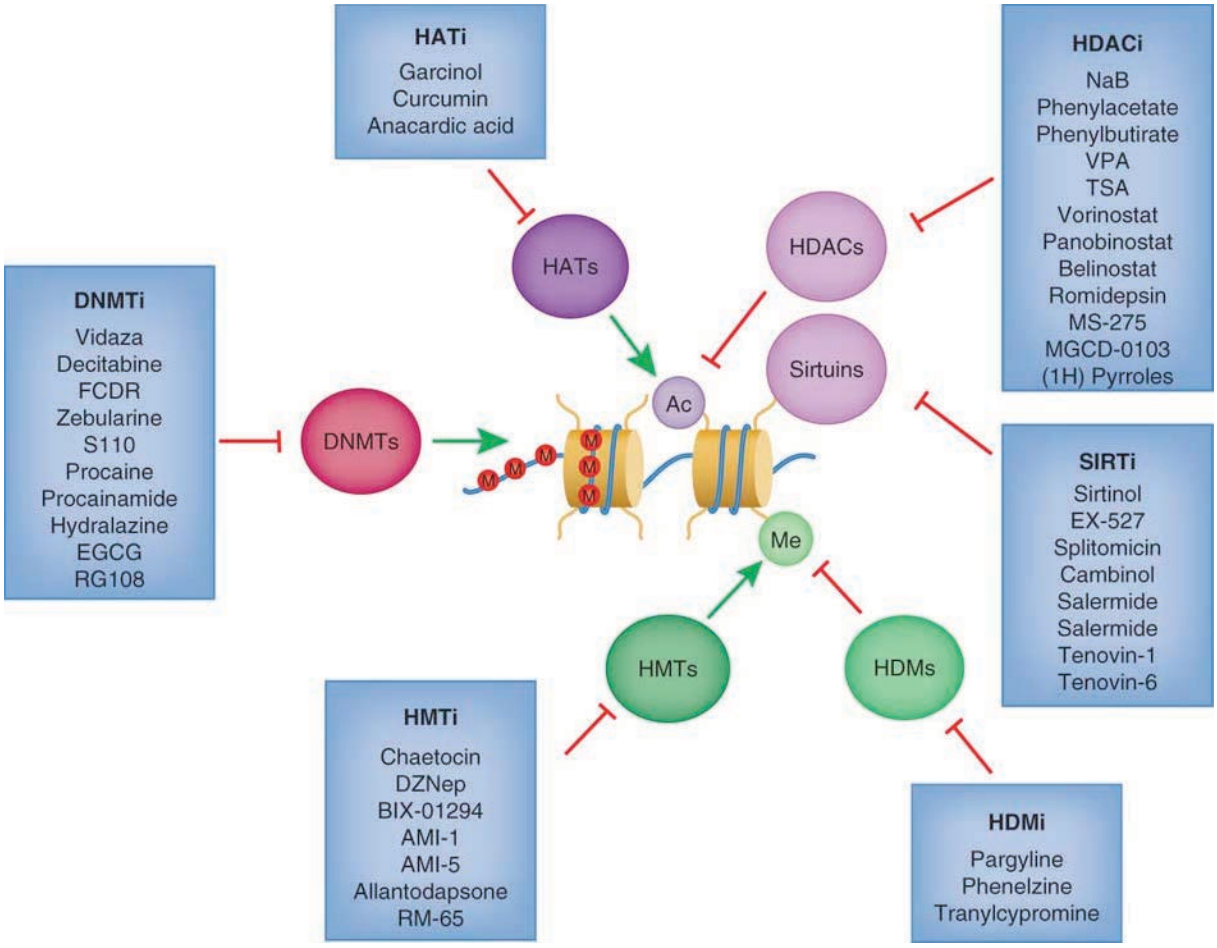


Figure 45: Drugs inhibiting enzymes involved in epigenetic modifications. This figure shows the most important epigenetic drugs classified depending on their particular epigenetic targets [671].

III.5.3 Inhibitors of histone acetylation and deacetylation

HATs have been implicated in several diseases such as cancer, asthma, COPD, Alzheimer's, and viral infections. Small-molecule inhibitors such as bisubstrate, garcinol, curcumin or anacardic acid have been identified but lack potency, specificity or the ability to penetrate the cell [679,680] (Figure 45). On the other hand, a large number of structurally diverse classes of HDAC inhibitors (HDACis) have been identified, such as hydroxamic acid-derived compounds (Trichostatin A (TSA), Suberoylanilide Hydroxamic Acid (SAHA)), short-chain fatty acids (sodium butyrate), benzamides, cyclic tetrapeptides and thiolates [681] (Figure 45). The classic HDACis TSA and SAHA inhibit class 1 and class 2 HDACs. HDACis are currently investigated for treatment of cancer, due to their antiproliferative activity, and mental disorders [666,682]. Moreover, these sort of drugs exhibit pleiotropic effects on a variety of cell functions, such as the regulation of the cell cycle, DNA recombination and repair, extrinsic and intrinsic apoptosis pathways, angiogenesis, autophagy and senescence, and the regulation of ROS concentration [682,683]. All the known HDACis block one class or several classes of HDACs and thus should have a global effect on gene expression. Surprisingly, comprehensive microarray gene expression experiments revealed that only a relatively small proportion of genes are up or downregulated following treatment with HDACis [684-686]. This can be explained by the fact that histone modifying enzymes, such as HDACs are targeted to specific genes and the inhibitor will only influence genes that are targets of HDACs. Moreover, it indicates the prevalence of non-transcriptional targets of HDACis [682,687].

III.5.4 HDACis as suppressors of inflammation

HDACs play important roles in the regulation of inflammation and immunity [644]. Several *in vivo* studies have shown the anti-inflammatory properties of HDACis for example in experimental colitis models in mice [688-691]. A study by Leoni *et al.* found that oral administration of SAHA to mice prior to LPS stimulation dose-dependently reduced circulating inflammatory cytokines TNFα, IL1β, IL6 and IFNγ [688]. Similar effects were observed *in vitro* in human peripheral blood mononuclear cells and mouse macrophages, where additionally the production of nitric oxide was suppressed. Further studies in different mouse models of experimental colitis indicate a protective effect of the HDAC inhibitors SAHA and valporic acid, as oral

administration ameliorated colonic proinflammatory cytokine expression and disease severity and thereby even prevent inflammation associated carcinogenesis [690,691].

Summary

In chapter III, we have discussed about the basics of genomic organization and gene expression. We have learned about the importance of histone proteins and the post-translational modification of their tails, which have a variety of functions in gene expression. Furthermore, we have discussed important studies, concerning the differential induction of gene expression by various stimuli. In this regard, we have learned that epigenetic mechanisms are able to separate genes in different clusters of expression and inducibility. We have focused on the knowledge of genetic and epigenetic regulation of AMPs. In this regard we have discussed transcription factors as well as HDAC enzymes and this was followed by a description of the enzymes involved in acetylation and deacetylation of histone and non-histone proteins. Finally, we have taken a look at drugs, which are able to inhibit enzymes involved in epigenetic modifications.

The epigenetic regulation of (inducible) gene expression opens up a whole new field of medical treatment options. As we learned epigenetic drugs are already on the market for cancer treatment and are in the process of being tested for the treatment of inflammatory disorders. In this regards, HDAC inhibitors present an interesting option for the prevention of inflammation, as the work of Glauben and Leoni demonstrated. We know that the dietary HDAC inhibitor butyrate induces AMP expression in the intestine, but we don’t know the mechanisms, by which acetylation and other possible epigenetic modification are involved in their expression. Therefore, we set out in this presented work to explore the influence of epigenetic regulatory mechanism on the expression of AMPs in the intestine, by the use of epigenetic drugs, such as HDAC inhibitors.

Chapter IV – Aim of the Thesis

Antimicrobial peptides are an important part of the innate immune system and vigorous defenders of the body's epithelial surfaces. Especially in the intestine, they are essential for the maintenance of homeostasis and balance with the local microbiota, and defense against intruding pathogens. They are not only capable to annihilate a multitude of different microorganism, but also are chemoattractants for adaptive immune cells and mediate wound healing and tissue regeneration. Their role in human pathologies has been widely described and deregulation of their expression or function can have detrimental effects on human health.

AMPs expression can be either constitutive or inducible and many internal and external stimuli have been described, which are capable to induce AMPs expression, in different tissues and cell types, via cellular signaling pathways. Still, the regulation of AMPs gene expression remains widely undiscovered. Few transcription factors involved in AMPs expression have been described, but are also in concordance with expression of INFs genes, such as the transcription factor NF-κB.

Besides signaling pathways and transcription factor activation, epigenetic mechanisms are major regulators of inducible gene expression. Chromatin remodeling and post-translational modifications of histones can impact the accessibility of promoters or enhancers, and the recruitment of cofactors. In this regard, enzymes, which mediate deacetylation of histones (but also cellular proteins) have been implicated in the expression of AMPs genes, and repression of INFs genes by previous studies.

The aim of the presented thesis was to decipher the epi/genetic components in the regulation of AMPs gene expression in the human intestine, with a special interest in regulation by de/acetylation. In the light of previous studies, regarding inhibition of histone deacetylases and inflammatory gene expression, we were additionally interested to explore the possibility of a disconnection of the AMP response from the inflammatory response via epigenetic regulatory mechanisms.

The set up of an *in vitro* model of intestinal epithelial cells, challenged with *E. coli* strains, as a stimulus to induce AMPs and INFs gene expression, was employed to study AMPs gene regulation.

Results

Chapter V – Results

The following chapter summarizes the main findings obtained during the course of the presented thesis project. This section is divided into two parts: In chapter V.1 the manuscript in preparation is presented, followed by a chapter of additional results in V.2.

The manuscript in preparation (V.1) describes the role of acetylation in the expression of antimicrobial peptides, in comparison to inflammatory genes. The results present the influence of histone deacetylase inhibitors on the expression of DEFB2 and IL8 genes, in the context of intestinal epithelial cell challenge with the commensal *E. coli* strain K12. Furthermore, results aiming to decipher the involvement of HDAC and HAT enzymes, histone marks, MAPK pathways and the transcription factor NF-κB, are presented.

The additional results section (V.2) illustrates results obtained with the pathobiontic *E. coli* strain LF82, which was used all along the experimental procedures, additionally to the K12 strain (V.2.1 and V.2.2). Furthermore, additional siRNA knockdown data are presented, concerning the involvement of HDAC (V.2.3) and HAT (V.2.4) enzymes.

V.1 Manuscript in preparation

Inhibition of histone deacetylases enhances expression of antimicrobial peptide genes upon a bacterial challenge

Natalie Fischer[1,2], Emmanuel Sechet[1,2], Robin Carl Friedman[1,2], Philippe J. Sansonetti[1,2,3,#] and Brice Sperandio[1,2,#]

[1]Unité de Pathogénie Microbienne Moléculaire, Institut Pasteur, 75724 Paris cedex 15, France

[2]INSERM U786, Institut Pasteur, 75724 Paris cedex 15, France

[3]Microbiologie et Maladies Infectieuses, Collège de France, 75231 Paris cedex 05, France

[#]Correspondence: brice.sperandio@pasteur.fr (B.S.), philippe.sansonetti@pasteur.fr (P.J.S.)

Running title: HDAC inhibition enhances expression of antimicrobial peptides

Keywords: intestinal mucosa, epithelial cells, antimicrobial peptides, epigenetic regulations, acetylation, *Escherichia coli*

Introduction

Remodeling of chromatin between relatively “open” and “closed” forms has a key role in epigenetic regulation of gene expression. Such remodeling results from modifying the structure of nucleosomes, the fundamental units of chromatin, which comprise approximately two turns of DNA wound around a histone octamer. A range of modifications of the amino-terminal “tails” of histone proteins are involved in this process, including acetylation, methylation, phosphorylation, poly-ADP ribosylation,

ubiquitinylation, sumoylation, carbonylation and glycosylation (1). These modifications can also occur within the “globular” domain of histones, which forms extensive contacts with the DNA. The consequence of such modifications on gene expression depends on the amino acid residues targeted and their close environment. Perturbed balance between these modifications leads to aberrant gene expression (2). Recent evidence proves the impact of histone modifications on regulation of the innate immune response and the expression of associated defence genes (3, 4).

Among histone modifications, acetylation and deacetylation play a crucial role in transcriptional regulation of genes (5). The acetylation status of histone proteins is determined by the opposing actions of histone acetyl-transferases (HATs) and histone deacetylases (HDACs). HATs add acetyl groups to the ε-amino group of lysine residues of nucleosomal histones, while HDACs remove those acetyl groups. In most of the cases, there is a positive correlation between the level of histone acetylation and transcriptional activity. Acetylation of histones by HATs promotes a relaxed structure of the chromatin by negating the positive charge interacting with negatively charged DNA, allowing transcriptional activation. Conversely, HDACs can act as transcription repressors, due to histone deacetylation, and consequently promote chromatin condensation.

In humans, 18 HDACs have been identified and classified, based on their homology to yeast HDACs (6). Class I HDACs (HDAC1, HDAC2, HDAC3 and HDAC8) are related to the yeast RPD3 deacetylase. Class II HDACs (HDAC4, HDAC5, HDAC6, HDAC7, HDAC9 and HDAC10) have homology with the yeast HDA1. All class I and II HDACs are zinc-dependent proteins and their enzymatic activity can be inhibited by compounds such as Trichostatin A (TSA) or Suberoylanilide Hydroxamic Acid (SAHA) (7). Members of the Class III, named Sirtuins, require NAD^+ for their enzymatic activity. Class IV HDAC is represented by HDAC11 and has conserved residues in the catalytic region shared by both class I and II enzymes (8). On the other hand, HATs are classified based on their cellular localization to nuclear HATs (type A) and cytoplasmic HATs (type B) (9). Several nuclear HATs have been identified, whereas only one cytoplasmic HAT has been described so far. Additionally, HATs have been devided into five families based on their primary structure homology. The three families that have been studied extensively are the

GNAT family (GCN5, PCAF), the MYST family (Tip60), and the well-known p300/CBP family (p300, CBP).

Despite their appellation, a huge number of non-histone proteins have been identified as substrates for both HATs and HDACs. Many of these proteins are transcription factors involved in the regulation of gene expression, including the transcription factor NF-κB, which regulates a wide range of host genes involved in the host innate immune response (10, 11). Reversible acetylation of the p65 subunit regulates diverse functions of NF-κB, including DNA binding and transcriptional activity, as well as its ability to associate with the sequester IκBα (12). Seven acetylated lysines have been identified within p65, including lysines K122, K123, K218, K221, K310, K314 and K315. The majority of these residues are acetylated by the HAT p300/CBP (13). For example, acetylation of K310 has been shown to be required for full transcriptional activity of NF-κB, but does not affect DNA binding (14). Conversely, several HDACs, including HDAC1, HDAC3 and SIRT1, have been found to specifically deacetylate p65 and thereby negatively regulate the transcriptional activity of NF-κB (12).

Most genes involved in the innate immune response are inducible genes, whose expression has to be highly regulated and must be able to be rapidly and specifically activated in response to diverse stimuli (15). This is the case at the human intestinal mucosal surface, where epithelial cells have to manage the expression of multiple genes, including antimicrobial and pro-inflammatory genes. These two groups of genes often have to be synchronously activated to protect the epithelium and keep commensal bacteria at bay and far from the surface. However, it is likely that such genes with different biological functions should have distinct requirements for regulation. Genes encoding pro-inflammatory mediators should be express at the appropriate level to avoid a detrimental effect on the tissue physiology. On the other hand, genes encoding antimicrobial peptides should be expressed at a basal level to provide suitable protection of the mucosa against the resident microbiota, or over-expressed under critical circumstances, such as infectious situations.

In this study, we conducted an in-depth analysis aiming to compare the regulation of the two classes of genes encoding antimicrobial peptides (DEFB2, DEFB3, LL37) and pro-inflammatory mediators (IL1B, IL8, CCL20) in human intestinal epithelial cells. Using the *Escherichia coli* K12 commensal bacteria as an inducer, we reveal

that induction of the DEFB2 antimicrobial gene expression is greatly enhanced upon inhibition of histone deacetylases enzymes, while transcription of the IL8 pro-inflammatory gene is not impacted. We show that this mechanism relies on an increased strength of the DEFB2 promoter, which ultimately leads to enhanced secretion of the DEFB2 peptide. We investigated the molecular mechanism underlying this observation at the chromatin level and highlight post-translational modifications of the histone H3 protein by acetylation and phosphorylation, at specific lysine and serine residues. We demonstrate that NF-κB is also acetylated on a specific lysine residue, in part by p300, and that both NF-κB and p300 mediates the enhanced induction of the DEFB2 gene expression upon inhibition of histone deacetylases. Finally, we identify additional genes of the antimicrobial defence and the epithelial restitution that exhibit a similar expression pattern. This work highlights existence of differential regulatory mechanisms occurring between antimicrobial and pro-inflammatory genes that take place at the epigenetic level and that are dependent on the acetylation process, in intestinal epithelial cells.

Materials and Methods

Antibodies

We used the rabbit polyclonal antibodies anti-NF-κB p65 subunit (SC-109, Santa Cruz), anti- NF-κB p65 acetyl K310 (ab52175, Abcam), anti-NF-κB p105/p50 (3035, Cell Signalling), anti-IκBα (SC-371, Santa Cruz), anti-histone H3 (ab1791, Abcam), anti-acetyl-histone H3 K9/14/18/23/27 (ab47915, Abcam), anti-acetyl-histone H3 K4 (07-539, Millipore), anti-acetyl-histone H3 K9 (ab4441, Abcam), anti-acetyl-histone H3 K14 (07-353, Millipore), anti-acetyl-histone H3 K18 (ab1191, Abcam), anti-acetyl-histone H3 K23 (07-355, Millipore), anti-acetyl-histone H3 K27 (ab4729, Abcam), anti-acetyl-histone H3 K56 (07-677-I, Millipore), anti-histone H4 (ab7311, Abcam), anti-acetyl-histone H4 K5/8/12/16 (06-866, Millipore), anti-acetyl-histone H4 K5 (07-327, Millipore), anti-acetyl-histone H4 K8 (07-328, Millipore), anti-acetyl-histone H4 K12 (07-595, Millipore), anti-histone H3 phospho-S10 (ab5176, Abcam), anti-phospho-SAPK/JNK (T183/Y185) MAPK (9251, Cell Signalling), and anti-actin (A2066, Sigma). We used the rabbit monoclonal antibodies anti-p38 MAPK (8690,

Cell Signalling), anti-phospho-p38 (T180/Y182) MAPK (4511, Cell Signalling), anti-p44/42 MAPK (4695, Cell Signalling), anti-phospho-p44/42 (T202/Y204) MAPK (4370, Cell Signalling). We used the mouse monoclonal antibody anti-KAT3B/p300 (ab14984, Abcam), the sheep antibody anti-mouse-IgG-POX (NXA931, GE Healthcare), and the goat antibody anti-rabbit-IgG-POX (GAR/IgG(H+L)/PO, Nordic Immunology).

Cell culture

The Caco-2 human intestinal epithelial cell line, subclone TC7 (16), was cultured with DME (Invitrogen) supplemented with 10% decomplemented Fetal Bovine Serum (FBS, Invitrogen), 1% non-essential amino acids (NEAA, Invitrogen), 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen), at 37°C and 10% CO_2. Cells were split two times per week, using Versene solution (Invitrogen).

Bacterial challenges

The *Escherichia coli* K12 bacterial strain was grown in LB medium (Sigma) at 37°C. For challenge experiments, cells were grown at confluence in 6-well plates (1.5×10^6 cells/well) for 48 h at 37°C and 10% CO_2. Bacterial challenges were performed using supplemented DME without antibiotics directly with over night bacterial cultures, at a multiplicity of infection (MOI) of 10 bacteria per cell, for indicated times. For experiments involving pharmacological inhibitors, cells were pretreated for 16 h over-night and washed with supplemented DME without antibiotics before infection.

Inhibitors

We used the pharmacological inhibitors Trichostatin A (T1952, Sigma), Suberoylanilide Hydroxamic Acid (SC-220139, Santa Cruz Biotechnology), Pargyline Hydrochloride (P8013, Sigma), 5-Azacitidine (A1287, Sigma), BMS-345541 (B9935, Sigma), C646 (SML0002, Sigma).

Transfections

The RNAi Max reagent (Invitrogen) was used to transfect cells with a final concentration of 25 nM siGENOME SMARTpool siRNAs (Thermo Scientific) targeting p65 (M-003533-02-0005), p300 (M-003486-04-0005), or control scramble siRNAs (D-001210-01-50). Transfections were performed in OptiMEM medium (Invitrogen)

supplemented with 1% NEAA and 5% FBS. Knockdowns were assessed after 48 h using qRT-PCR and immunobloting analysis.

qRT-PCR

RNA was isolated using the RNeasy Mini kit and the RNase free DNase kit (Qiagen). RT-PCR reactions were performed over night on RNA samples using the SuperScript II reverse transcriptase (Invitrogen) and the oligo(dT)18 primers (Thermo Scientific), as recommended by the suppliers. Gene-specific primers were designed and purchased from Sigma (DEFB2 exon GCCATGAGGGTCTTGTATCTC / TTAAGGCAGGTAACAGGATCG, DEFB2 intron/exon AGCAGTGCCAGTTTCCATGTCA / TAAATCCGCAGCGGGCTTCTTT, DEFB3 TTTGGTGCCTGTTCCAGGTCAT / GCCGCCTCTGACTCTGCAATAATA, LL37 AAGGAAGCTGTGCTTCGTGCTA / AATCCTCTGGTGACTGCTGTGT, IL1B TACGATCACTGAACTGCACGCT / TCTTTCAACACGCAGGACAGGT, IL8 exon AAGAAACCACCGGAAGGAACCA / ATTTCTGTGTTGGCGCAGTGTG, IL8 intron/exon ACTGAGGTCAAGGGCTAGGAGAAT / AGCTCTGCCAGCTACTTCCTTT, CCL20 AAGAGTTTGCTCCTGGCTGCTT / GCAGTCAAAGTTGCTTGCTGCT, B2M ATTGCTATGTGTCTGGGTTTCA / AAGACAAGTCTGAATGCTCCAC). The qRT-PCR reactions were carried out in a 20 µl final volume containing 8 µl of cDNA (diluted at 1/100), 2 µl of primers (0,2 µM each), and 10 µl of Power SYBR Green mix (Applied Biosystems). Reactions were run on an ABI 7900HT instrument (Applied Biosystems) with conditions of the recommended universal thermal cycling parameters. Each sample reaction was run in duplicate on the same plate. Relative gene expression quantification was performed using the comparative Ct method. Data were normalized to the β-2-microglobulin (B2M) housekeeping gene expression.

ELISA

We used the Enzyme-linked Immunosorbant Assay (ELISA) kits for DEFB2 (900-K72, PeproTech), and IL8 (900-K18, PeproTech). Absorbance was measured on a M200PRO fluorimeter (Tecan).

Immunoblotting

Total cell lysates were harvested by removing growth medium and adding NP40 lysis buffer [25 mM Tris HCl (pH 7.5), 1 mM EDTA, 0.1 mM EGTA, 5 mM $MgCl_2$, 1% NP40, 10% Glycerol, 150 mM NaCl] supplemented by a cocktail of protease inhibitors [Sodium Orthovanadate (Sigma), 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride (Sigma), COMPLETE (Roche)]. Samples were diluted with Blue juice buffer [1M Tris HCl, 20% Glycerol, 6% SDS, 0.02% Bromophenol Blue, 10% β-Mercaptoethanol] and boiled for 5 min. Denatured proteins were loaded on 7.5%, 10% or 12% acrylamide Mini PROTEAN TGX precast gels (BioRad). Separated proteins were transferred onto a PVDF membrane using the iBlot Gel Transfer System (Invitrogen). Membranes were blocked with 3% Albumin from Bovine Serum (BSA, Sigma) or 5% milk (Regilait), at room temperature, prior to incubation with primary antibodies, over night at 4°C, in 1% BSA or 5% milk. Incubation with the secondary horseradish peroxidase-conjugated IgG antibody was performed at room temperature. Blots were developed using the SuperSignal West Dura Extended Duration Substrate solution (Thermo Scientific) and the ChemiDoc XRS System (BioRad).

Cytotoxicity measurement

Cytotoxic effects of bacterial infection and pharmacological inhibitor treatment were evaluated by measurement of lactate dehydrogenase (LDH) release, using the CytoTox 96 non-radioactive Cytotoxicity Assay (Promega).

RNA sequencing

RNA libraries were created with Illumina TruSeq stranded PolyA+ mRNA kits sequenced on 2 Illumina HiSeq lanes, 3 samples multiplexed per lane, with paired-end 50 base pair reads. Reads were mapped to human genome hg19 (GRCh37) using TopHat 2.0.11, with Gencode 19 human genes as a transcriptome guide and maximum of 50 multihits. Reads mapping to Gencode genes were counted with the featureCounts software of the Subread package. Read counts were normalized using the DESeq 1.14 estimateSizeFactors function. Genes having less than 10 reads in any condition were filtered out, and remaining transcripts had a pseudocount of 5 added to down-weight poorly expressed transcripts in fold-change calculations.

Results

Antimicrobial and pro-inflammatory genes are induced in cells challenged with commensal bacteria

To analyse the expression of inducible genes involved in the innate immune response in intestinal epithelial cells exposed to commensal bacteria, such as the genes encoding antimicrobial peptides (DEFB2, DEFB3, LL37) (Fig. 1A) and pro-inflammatory mediators (IL1B, IL8, CCL20) (Fig. 1B), we challenged the human colonic epithelial Caco-2 cells, subclone TC7, with the *Escherichia coli* K12 strain. Experiments were performed on confluent cell monolayers using a ratio of ten bacteria per cells. RNA was harvested at different time points after challenge and analyzed by qRT-PCR for expression of the selected set of genes.

In non-challenged cells, we observed a basal expression of the beta-defensins DEFB2, DEFB3, cathelicidin LL37, interleukins IL1B, IL8, and chemokine CCL20 genes (unpublished data). Challenge of cells with *E. coli* was followed by strong transcriptional induction of the DEFB2, IL8, IL1B, and CCL20 genes, whereas the expression of DEFB3 and LL37 genes was only weakly induced throughout the experiment (Fig. 1A-B). Cellular viability, as assessed by lactate dehydrogenase assay, remained the same for cells challenged or not (unpublished data). Collectively, these results demonstrate that major genes encoding antimicrobial peptides and pro-inflammatory mediators are expressed and induced together in intestinal epithelial cells exposed to commensal bacteria.

Induction of antimicrobial peptide gene expression is enhanced upon inhibition of histone deacetylases

To investigate the epigenetic mechanisms taking part in the induction of genes of the innate immune response, we tested the impact of inhibition of several chromatin modifying enzymes on the expression of antimicrobial and pro-inflammatory genes. Dose response assays were carried out over-night on confluent cell monolayers with inhibitors of DNA methylation (Azacytidine), histone demethylation (Pargyline), and

histone deacetylation (Trichostatin A, Suberoylanilide Hydroxamic Acid). The inhibitor-containing medium was removed, and *E. coli* challenges were performed to induce expression of innate immune genes. RNA was extracted at 2h after challenge and analyzed by qRT-PCR for expression of genes encoding antimicrobial peptides and pro-inflammatory mediators.

In our model, we found that inhibition of DNA methylases or histone demethylases had no effect on the induction of expression of the set of antimicrobial and pro-inflammatory genes, whatever the inhibitor concentration used (unpublished data). In contrast, inhibition of histone deacetylase enzymes impacted the induction of expression of the two classes of genes differently (Fig. 2 and S1). Induction of antimicrobial DEFB2 and LL37 gene expression was significantly enhanced in a concentration dependent manner (Fig. 2A), whereas induction of pro-inflammatory gene expression was insensitive to the inhibitor (Fig. 2B). Upon treatment with 5 µM TSA prior to infection, the bacteria-induced expression of DEFB2 was additionally increased more than 1000-fold compared with less than 100-fold without inhibitor treatment (Fig. 2A). In contrast, induction of the IL8 gene expression was similar for all tested TSA concentrations (Fig. 2B). Interestingly, this pattern of expression upon inhibition of histone deacetylases was also observed in non-challenged cells, as previously described for the expression of the cathelicidin LL37 gene, in colonic cells treated with the histone deacetylase inhibitor sodium butyrate (Fig. 2A) (17). As a control, cellular viability was only affected for cells treated with the highest concentration of pharmacological inhibitor, as assessed by lactate dehydrogenase assay (unpublished data, Fig. S1). Together, these results show that inhibition of histone deacetylase enzymes has a differential impact on the expression of genes encoding antimicrobial peptides and pro-inflammatory mediators in intestinal epithelial cells.

Strength of the DEFB2 gene promoter, maturation of DEFB2 pre-messengers, and translation of the DEFB2 messengers are increased upon inhibition of histone deacetylases

To further characterize the enhanced induction of the DEFB2 gene expression observed upon inhibition of histone deacetylase enzymes, we proceeded to a

detailed analyze of the transcription of this gene after treatment of confluent cells with 5 µM TSA prior to *E. coli* challenge.

To study the strength of the DEFB2 gene promoter, we quantified DEFB2 pre-mRNA at different time points by qRT-PCR, using a couple of primers matching at the junction of an intron and an exon of the gene sequence (Fig. 3A). Kinetic and magnitude of DEFB2 pre-mRNA transcription were significantly increased, with more than 1 Log of difference, within the first hour post-challenge in TSA treated cells compared to non-treated cells. This indicates that inhibition of histone deacetylases increased the strength of the DEFB2 gene promoter. Interestingly, this difference was dampened at the later time point and returned to normal infection-induced level at 6h post-challenge, suggesting that a modification occuring at the promoter moderated its activity. By comparison, IL8 pre-mRNA transcription was not influenced by histone deacetylase inhibition (Fig. 3A).

To analyze the maturation of DEFB2 gene pre-mRNA transcripts, we quantified the amount of mature DEFB2 mRNA by qRT-PCR, using a couple of primers matching in the first exon of the gene sequence (Fig. 3A). As observed for DEFB2 pre-mRNA, the kinetic and magnitude of DEFB2 mRNA detection were significantly increased in TSA treated cells compared to non-treated cells, reflecting the splicing process of pre-mRNA into mRNA. Conversely, IL8 mRNA levels remained unchanged upon inhibitor treatment (Fig. 3A).

To investigate translation of DEFB2 mRNA into protein, cells were challenged with *E. coli* for 6h. Bacteria were then killed by gentamicin treatment, and DEFB2 peptide secreted in the supernatant of cells was quantified after 24h, by ELISA (Fig. 3B). In supernatants of non-challenged cells, the DEFB2 peptide was detected in small amounts after inhibition of histone deacetylases alone, showing that induction of the DEFB2 gene expression observed subsequently to the TSA treatment (Fig. 2A) was enough to stimulate the peptide synthesis and secretion. In supernatants of cells challenged with *E. coli*, dosage of the DEFB2 peptide revealed a concentration reaching 10^4 pg/ml for TSA treated cells, after 24h, compared to 10^2 pg/ml for non-treated cells. By comparison, secretion of the interleukin IL8 was found to be identical in both conditions (Fig. 3B). As a control, cellular viability was tested and found similar for challenged cells treated or not with TSA, as assessed by lactate dehydrogenase assay (unpublished data). Collectively, these results show that

inhibition of histone deacetylase enzymes enhances activity of the DEFB2 gene promoter and the DEFB2 gene transcription, increasing ultimately the DEFB2 peptide synthesis and secretion.

Phosphorylation of histone H3 is enhanced upon inhibition of histone deacetylases

Based on these observations, we sought to determine the molecular mechanism governing the enhanced induction of the DEFB2 gene expression upon inhibition of the histone deacetylases. Several studies have suggested that accessibility of transcription factors to chromatin on the promoter of genes of the innate immune response requires MAPK-induced phosphorylation of histone H3 at the serine 10 residue (3, 18). We therefore investigated the effect of inhibition of histone deacetylases on histone H3 phosphorylation and MAPKs activation in *E. coli* challenged cells (Fig. 4).

Strikingly, using an antibody against histone H3 phosphorylated at serine 10 residue, we observed that TSA treatment induced a strong increase of this phosphorylation mark, in non-challenged as well as challenged cells (Fig. 4).

By immunoblot, using an antibody detecting phosphorylated T202/Y204 residues on Erk, we found that TSA treatment alone reduced the basal level of Erk phosphorylation in non-challenged cells (Fig. 4). This TSA-dependent decrease of Erk phosphorylation was reverted in cells challenged with *E. coli*, as shown by the increased level of Erk phosphorylation observed in treated and challenged cells, as soon as 1 hour post-challenge (Fig. 4).

Conversely, the basal level of p38 phosphorylation was not impacted by the TSA treatment in non-challenged cells, as assessed by the use of an antibody detecting phosphorylated T180/Y182 residues on p38 (Fig. 4). Increased form of phosphorylated p38 was observed upon *E. coli* challenge, in TSA treated as well as non-treated cells, with a maximum reached between 1-3h post-challenge (Fig. 4). Together, these data show that upon inhibition of histone deacetylases, the Erk and p38 MAPKs are activated upon a challenge with *E. coli*, and that phosphorylation of histone H3 at the serine 10 residue is dramatically enhanced, suggesting activation of an alternative pathway in charge of this histone modifying process.

Inhibition of histone deacetylases increases acetylation of specific histone H3 lysine residues

Histone modifications exert their effects via two main mechanisms. The first involves the electrostatic modification directly influencing the overall structure of chromatin, the second involves the access modification regulating the binding of transcription factors and co-regulators. Among these modifications, besides phosphorylation, also histone acetylation has the potential to disrupt the interaction between histones and DNA, thereby facilitating DNA access by transcription factors. Therefore, we investigated the consequence of histone deacetylase inhibition on acetylation level of the histone H3 and H4 proteins at different lysine residues.

By immunoblot, using specific antibodies, we identified two classes of lysine residues that were differentially impacted by the TSA treatment, in non-challenged cells as well as *E. coli* challenged cells. The first class included lysines constitutively acetylated and poorly affected by the TSA treatment, such as the residues H3K4, H3K14, H3K18, H3K23, H4K5, H4K8, H4K12, and H4K16 (Fig. 5 and Fig. S2). The second class included lysines greatly acetylated upon the TSA treatment alone, like the residues H3K9, H3K27, and H3K56 (Fig. 5 and Fig. S2). Together, these results indicate that single lysine residues from the histone H3 and H4 are differently impacted upon inhibition of histone deacetylase enzymes.

NF-κB mediates the enhanced induction of DEFB2 gene expression upon inhibition of histone deacetylases

A current model suggests that post-translational modifications of histone H3 by phosphorylation at the serine 10 residue, and acetylation of surrounding lysine residues, accounts for a histone structure that favors the binding of chromatin-remodeling enzymes, which increase the promoter accessibility for the NF-κB transcription factor on a specific set of genes of the innate immune response (18). The promoter of DEFB2 as well as the promoter of IL8 possess NF-κB binding sites (19, 20). We therefore investigated the role of this transcription factor in the induction of expression of these two genes upon inhibition of histone deacetylase enzymes.

Using a pool of siRNA to target the expression of the p65 subunit, we found that transcription of the DEFB2 as well as the IL8 gene was dependent on NF-κB in TSA

treated as well as non-treated cells challenged with *E. coli* (Fig. 6A). In both conditions, upon an efficacy of p65 silencing reaching 80 %, expression of the DEFB2 and IL8 genes was reduced at least 10 fold compared to cells transfected with the scramble siRNA negative control.

To confirm these results by an alternative approach, we tested the impact of the chemical inhibitor BMS-345541 on the expression of the DEFB2 and IL8 genes in treated and challenged cells. This chemical is a specific inhibitor of the NF-κB pathway targeting the inhibitor kappa B kinase (IKK), the kinase complex that controls activation of the NF-κB transcription factor (21). Without BMS-345541 treatment, DEFB2 expression was enhanced more than 1 Log in TSA treated cells compared to non-treated cells, as soon as 1 hour post-challenge, as previously observed (Fig. 3A and 6B). Strikingly, expression of the DEFB2 and IL8 genes was completely abolished upon treatment by the BMS-345541 inhibitor, both in cells treated or non-treated with TSA (Fig. 6B). As a control, cellular viability was not affected in all tested conditions, as assessed by lactate dehydrogenase assay (unpublished data). Collectively, these results demonstrate that NF-κB is essential for induction of the DEFB2 and IL8 gene expression, as well as for the enhanced induction of the DEFB2 gene expression observed upon inhibition of the histone deacetylase enzymes.

NF-κB is acetylated upon inhibition of histone deacetylases

The NF-κB transcription factor exists in homo- or hetero-dimeric complexes consisting of different members of the REL family of proteins. In resting cells, p50-p65 is present in the cytoplasm in an inactive form, bound to inhibitory proteins known as IκBs, including IκBα. Upon stimulation, such as a bacterial challenge, the IκBs are phosphorylated by IKK, ubiquitinated and degraded. Degradation of IκBα results in the release of the p50-p65 dimer, which translocates into the nucleus, followed by specific upregulation of gene expression (11). Since we found NF-κB to be essential for the induction of DEFB2 gene expression, we sought to determine the impact of inhibition of histone deacetylases on the NF-κB complex. Using an antibody detecting the p65 protein, we found that TSA treatment had no significant impact on the protein level of this subunit of NF-κB, in non-challenged cells as well as in *E. coli*

challenged cells (Fig. 7). Conversely, we detected a decreased level of the p50 protein and its precursor p105 upon TSA treatment, as assessed by the use of an antibody recognizing the two forms of the protein (Fig. 7). Interestingly, we observed the degradation of IκBα in non-challenged cells upon TSA treatment (Fig. 7), providing evidence for activation of the NF-κB pathway by this histone deacetylase inhibitor. This observation could explain the induction of the DEFB2 gene expression and DEFB2 peptide secretion detected in non-challenged cells subsequently to a TSA treatment (Fig. 2A and Fig. 3B). In comparison, we observed the resynthesis of IκBα in challenged cells, as soon as 2 hours post-challenge (Fig. 7). This reappearance of the NF-κB inhibitor could be an explanation for the observed decrease of DEFB2 pre-mRNA synthesis after 3h post-challenge (Fig. 3).

Recent studies indicate that post-translational modifications of NF-κB, especially of the p65 subunit, play a critical role in fine-tuning the activity of this transcription factor, adding another layer of complexity to the transcriptional regulation of NF-κB. Among them, reversible acetylation of p65 regulates diverse functions of this transcription factor, including its ability to associate with IκBα (12). To investigate whether NF-κB is post-translationaly modified by acetylation upon inhibition of histone deacetylases, we used an antibody detecting acetylation of the lysine K310 residue of the p65 subunit. By immunoblot, we found that TSA treatment dramatically induced acetylation of p65 on this residue (Fig. 7). Acetylation of this lysine residue was only detected within the first hour following the treatment of cells with TSA, suggesting a highly dynamic reversibility of the acetylation mark. Together, these results show that inhibition of histone deacetylase enzymes activates the NF-κB pathway, as suggested by the degradation of IκBα, and leads to a reversible acetylation of the p65 subunit.

Histone acetyltransferase p300 takes part in NF-κB acetylation and mediates induction of DEFB2 gene expression

Acetylation mostly occurs in the nucleus, where most of the enzymes mediating this modification reside. These enzymes include histone acetyltransferases and histone deacetylases, which mediate the addition or removal of the acetyl group to and from lysine residues. Recently, it has been shown that the p65 subunit of NF-κB is

acetylated at the lysine K310 residue by the histone acetyltransferase p300, and that acetylation of this lysine is required for the transcriptional activity of NF-κB (14, 22). We therefore investigated the role of p300 on acetylation of the p65 subunit of NF-κB and the induction of the DEFB2 gene expression.

Using a pool of siRNA to target expression of the p300 histone acetyltransferase in cells challenged with *E. coli*, we found that the p65 protein was less acetylated on the lysine K310 residue, compared to cells transfected with the scramble siRNA negative control (Fig. 8A). Interestingly, this observation correlated with a significantly decreased transcription of the DEFB2 gene, upon p300 knockdown (Fig. 8B). This result was confirmed by an alternative approach, using the C646 chemical inhibitor, which is specific for the p300 histone acetyltransferase (Fig. 8C) (23). Dose response assays showed that transcription of the DEFB2 gene was reduced more than 1 log at the highest concentration of the C646 inhibitor, as compared to non-treated cells. On the other hand, expression of the IL8 gene was less impacted by the decreased amount or activity of the p300 histone acetyltransferase (Fig. 8B-C). As a control, cellular viability was not affected in all of these conditions, as assessed by lactate dehydrogenase assay (unpublished data). Collectively, these data demonstrate that the histone acetyltransferase p300 is involved in the acetylation process of the p65 subunit of NF-κB in our model, and mediates induction of the DEFB2 gene expression upon a bacterial challenge.

Expression of additional genes from the antimicrobial defence and epithelial restitution is enhanced upon inhibition of histone deacetylases

We finally hypothesized that the enhanced induction of the DEFB2 gene expression observed upon inhibition of histone deacetylase enzymes might reflect a global and differential regulatory mechanism existing between genes from the antimicrobial defence and the inflammation process. We therefore investigated whether other genes from the innate immune response exhibit a pattern of expression similar to the DEFB2 gene upon inhibition of histone deacetylases. For this purpose, we performed a transcriptomic analysis of cells treated or not with TSA and challenged with *E. coli*. RNA was extracted from cells and analyzed by RNA sequencing, using the Illumina technology. The fold-change calculations representative of the gene expression were

determined and presented on a scatter-plot (Fig. 9A). Among all the genes, we specifically focused our attention on those from the innate immune response (Fig. 9B-C). Validation of RNAseq data was performed on a set of genes, using qRT-PCR (unpublished data). From this cluster of genes, we identified a total of 58 genes whose expression was significantly modulated in cells upon the *E. coli* challenge.

Interestingly, we identified 19 genes whose expression was enhanced more than one Log2 in cells treated with TSA compared to non-treated cells, including the DEFB2 gene (Fig. 9B). Those were genes belonging to the antimicrobial defence, such as PGLYRP1, PGLYRP2, PGLYRP3, and PGLYRP4 encoding the four human peptidoglycan recognition proteins, which target bacterial membranes, PLA2G7 encoding the phospholipase A2, which hydrolyses bacterial membrane phospholipids, and LCN2 encoding the lipocalin iron sequestration protein. Remarkably, besides genes from the antimicrobial defence, expression of other genes belonging to the epithelial restitution, such as TGFB2, TSLP, and IL7, was also enhanced upon inhibition of histone deacetylases (Fig. 9B). TGFB2 is a member of the transforming growth factor beta family of cytokines involved in the promotion of the intestinal homeostasis. TSLP is a hemopoietic cytokine promoting T helper type 2 cell responses that are associated with immunity to moderate intestinal inflammation. IL7 is produced locally by intestinal epithelial cells, and may serve as a regulatory factor for intestinal mucosal lymphocytes. By comparison, expression of pro-inflammatory genes was quite similar in challenged cells treated or not with TSA, as observed for the IL1B and IL8 gene and most of genes encoding chemokines and cytokines (Fig. 9C). Collectively, these results show that expression of many genes from the antimicrobial defence and the epithelial restitution is enhanced upon inhibition of histone deacetylase enzymes, whereas expression of pro-inflammatory genes is only minimally impacted.

Discussion

The human intestinal epithelium achieves a protective barrier function between the host and its microbiota. This barrier protects against invasion and systemic dissemination of colonizing microorganisms. Among the numerous factors that

participate in establishment and maintenance of epithelial homeostasis, antimicrobial peptides play an important regulatory role. They are ubiquitously expressed by epithelial cells throughout the intestinal tract and keep in check the resident and transient bacterial populations (24). These effectors participate in the innate immune response to commensals under stady-state conditions, a situation of tolerance actively maintained by commensals themselves (25). Epithelial cells mediate and orchestrate this dialogue mainly through signal transduction pathways, which on one hand result in activation of histone modifying enzymes and remodeling complexes (26). These pathways induce expression of hundreds of genes with different functions and therefore different regulatory requirements (27). Because these genes are induced by the same pathways, their expression has to be regulated by gene-specific manners rather than signal-specific mechanisms. As such, in macrophages, genes encoding pro-inflammatory mediators are transiently inactivated to limit tissue damage, whereas genes encoding antimicrobial peptides, which do not affect tissue physiology remain inducible to provide continuous protection (28). This process is an adaptive element of the innate immune response based on epigenetic mechanisms.

One link between bacterial sensing and its effect on histones has been described for the MAPKs cascades, whose activation leads to phosphorylation of histone H3 on the serine 10 residue (H3S10). Both, the Erk and p38 kinases have been shown to activate the effector kinases MSK1 and MSK2 that directly phosphorylate H3S10 at the promoter of activated genes (29). We observed occurrence of this histone mark in cells challenged with the *E. coli* commensal bacteria. But unexpectedly, we also found a strong and durable phosphorylation of H3S10 in non-challenged cells upon inhibition of the histone deacetylase enzymes by TSA treatment. This occurred without significant activation of the MAPKs Erk and p38, as observed by their phosphorylation level. Therefore, we can speculate that the TSA inhibitor itself, or its inhibitory action on the histone deacetylase enzymes, are able to activate an alternative pathway in charge of the H3S10 phosphorylation. One potential candidate is the IκB kinase (IKK) pathway that can mediate phosphorylation of H3S10 and activate transcription of NF-κB-responsive genes (30, 31). IKK phosphorylates and targets the NF-κB-sequester IκBs regulatory proteins for proteasomal degradation. In agreement with this hypothesis, we observed a decrease in the protein level of IκBα upon TSA treatment in non-challenged cells, highlighting an activation of the IKK

pathway that might promote H3S10 phosphorylation. Furthermore, the current hypothesis is that phosphorylation of H3S10 is a predisposing mark for histone acetylation, a mark for active transcription (32). As we observed a strong increase in the acetylation of the H3K9, H3K27, and H3K56 residues upon TSA treatment, it is also tempting to speculate about the role of these modifications in the enhanced induction of the antimicrobial peptide gene expression. Whether these histone phosphorylation and acetylation marks occur differently at the DEFB2 and IL8 promoters will need to be deeply investigated to determine whether there is a correlation between their appearance and the expression of antimicrobial peptide encoding genes at varying degrees.

Phosphorylation of H3S10 and acetylation of H3 surrounding lysine residues are highlighted in a current model as discrete modifications promoting chromatin remodeling at the promoter of specific genes of the innate immune response, which allow a precise recruitment of NF-κB (18). Besides this chromatin-conditioned control, the NF-κB activity is subjected to other regulatory mechanisms. Among these, acetylation of specific lysine residues of the NF-κB subunits play distinct roles in the regulation of its DNA-binding ability, its transcriptional activity and duration of its actions (11). Interestingly, these post-translational modifications resemble not only to those amending histone proteins, but also shares the same key enzymes, such as HATs and HDACs. Recent studies showed that inhibition of HDAC enzymes by TSA treatment prolonged the presence of the p65 NF-κB subunit in the nucleus and enhanced its binding activity to the DNA, possibly by its increased acetylation (33, 34). We observed a strong acetylation of the p65 subunit on the lysine K310 residue upon inhibition of HDAC enzymes in our model. Thus, it is tempting to hypothesize that the increased acetylation of NF-κB strengthens its nuclear presence and activity, which would explain, to a certain extent, the enhanced basal expression as well as increased induction of the DEFB2 gene expression observed upon TSA treatment and *E. coli* challenge. Moreover, several studies have shown that upon DNA binding, NF-κB can recruits HATs to the target gene promoter where these enzymes change the acetylation profiles of histone proteins and function as co-activators for gene transcription (35, 36). Among them, p300 has been shown to interact with the transcriptional activation domain of p65. As we demonstrated that p300 takes part in the NF-κB p65 subunit acetylation process and in the induction of the DEFB2 gene

expression, it would be of interest to investigate whether p300, p65, and p65 acetylated on the lysine K310 residue are recruited to a same extent at the DEFB2 and IL8 gene promoters. This would give more insight into the role of the transcription factor NF-κB, the HAT/co-activator p300, and the acetylation status of the p65 subunit as well as histone proteins, in the differential induction of the DEFB2 and IL8 gene expression observed upon HDACs inhibition.

Several HDAC inhibitors have already been evaluated as therapeutic compounds with activities in cancer therapies (37). Recently, molecules derived from those inhibitors have been shown to have applications beyond cancer therapies, based on their additional properties (38). Apart from applications in oncology, a considerable research effort has been aimed at evaluating the potential of these inhibitor molecules as therapeutics for neurodegenerative disorders, cardiac hypertrophy, and asthma. However, except for HIV, hepatitis and malaria infection, the therapeutic potential of HDAC inhibitors has not been largely investigated for treatment of infectious diseases. One example is the HDAC inhibitor sodium butyrate, which can stimulate production of the endogenous cathelicidin antimicrobial peptide in the intestine and promote the clearance of the *Shigella* enteropathogen, leading to clinical improvement of chronic infection (39). Along the same line, we have demonstrated the properties of the HDAC inhibitor TSA to enhance induction of the DEFB2 gene expression without impacting the IL8 basal transcription upon a bacterial challenge. This observation highlights the possibility to disconnect the expression of antimicrobial peptide genes from those encoding pro-inflammatory mediators at the epigenetic level. Moreover, we found that inhibition of HDAC chromatin-modifying enzymes has a positive impact on the induction of expression of additional genes from the epithelial defence and restitution pathways in response to a bacterial challenge, without modifying pro-inflammatory gene expression. This suggests a potential role in immune therapy for these inhibitor molecules through epigenetic modifications. Understanding the coordinated interplay between epigenetic regulation, gene expression, and environment, will allow the translation of this fundamental knowledge into the development of innovative pharmacoepigenetic treatments.

Acknowledgments

The research leading to these results has received funding from the European Union Seventh Framework Programme under the Grant Agreement EIMID ITN No 264388. Philippe J. Sansonetti is supported by the European Research Councli (HOMEOEPITH project), and by the Howard Hughes Medical Institute. The authors have no conflicting financial interests.

Figure Legends

Figure 1. Expression of antimicrobial and pro-inflammatory genes upon challenge with *E. coli*. Transcriptional expression of genes encoding the antimicrobial peptide beta-defensin DEFB2 and DEFB3, the cathelicidin LL37 **(A)**, and the pro-inflammatory mediators interleukins IL1B and IL8, and the chemokine CCL20 **(B)**, in cells challenged with the *Escherichia coli* K12 commensal strain. After mRNA extraction and RT reactions, qRT-PCR was performed on each sample, for each time point, with specific primers to determine the relative expression of genes using the comparative Ct method. Values are presented on a logarithmic scale as the ratio of gene expression in challenged cells compared to non-challenged cells. Experiments were performed at a MOI of 10 bacteria per cell. N = 3 independent experiments. Error bars represent the SD.

Figure 2. Expression of antimicrobial and pro-inflammatory genes upon inhibition of histone deacetylases. Transcriptional expression of the DEFB2, DEFB3, and LL37 antimicrobial genes **(A)**, and IL1B, IL8, and CCL20 pro-inflammatory genes **(B)**, in cells treated for 16 hours with increasing concentrations of trichostatin A (0-50 µM TSA), and challenged for 2 hours with the *E. coli* K12 strain. After mRNA extraction and RT reactions, qRT-PCR was performed on each sample with specific primers to determine the relative expression of genes using the comparative Ct method. Values are presented on a logarithmic scale as the ratio of gene expression in treated and challenged cells (black bars), or treated and non-

challenged cells (white bars), compared to non-treated and non-challenged cells. Experiments were performed at a MOI of 10 bacteria per cell. N = 3 independent experiments. Error bars represent the SD. *, P < 0,01 for treated cells, challenged or not, compared to non-treated and non-challenged cells.

Figure 3. Expression of the beta-defensin DEFB2 and the interleukin IL8 at the pre-mRNA, mRNA, and protein levels, upon inhibition of histone deacetylases. **(A)** Kinetic of expression of the DEFB2 and IL8 genes in cells treated or non-treated for 16 hours with 5 µM TSA, and challenged with the *E. coli* K12 strain. After RNA extraction at each time point and RT reactions, qRT-PCR was performed on each sample with specific primers to determine the relative expression of pre-mRNA (dashed lines) and mRNA (solid lines) of genes using the comparative Ct method. Values are presented on a logarithmic scale as the ratio of gene expression in treated and challenged cells (black lines), or non-treated and challenged cells (grey lines), compared to non-treated and non-challenged cells. Experiments were performed at a MOI of 10 bacteria per cell. N = 3 independent experiments. Error bars represent the SD. **(B)** ELISA measurement of the amount of DEFB2 and IL8 proteins detected in supernatants of cells treated or non-treated for 16 hours with 5 µM TSA, and challenged or non-challenged for 6 hours with the *E. coli* K12 strain. Bacterial challenges were stopped at 6 hours by addition of gentamicin, and supernatants were collected 24 hours after the beginning of the challenge. Experiments were performed at a MOI of 10 bacteria per cell. Values are presented on a logarithmic scale in picogram of protein per milliliter. N = 3 independent experiments. NC: non-challenged cells ; K12: cells challenged with the *E. coli* K12 strain. Black bars: 5 µM TSA treated cells ; grey bars: non-treated cells. Error bars represent the SD. *, P < 0,01 for treated cells compared to non-treated cells.

Figure 4. Phosphorylation of MAPKs Erk and p38, and histone H3 on serine 10 residue, upon inhibition of histone deacetylases. Immunoblot analysis of the phosphorylation mark of the Erk1/2 and p38 MAPKs, and the histone H3 protein on serine 10 residue, in cells treated for 16 hours with 5 µM TSA, and challenged with the *E. coli* K12 strain. After cell lysis at the indicated time points, western-blots were

performed using specific antibodies directed against proteins or post-translational modification marks. Experiments were performed at a MOI of 10 bacteria per cell. N = 2 independent experiments. NC: non-challenged cells ; K12: cells challenged with the *E. coli* K12 strain. “P” prefix: phosphorylation.

Figure 5. Acetylation of histone H3 lysine residues upon inhibition of histone deacetylases. Immunoblot analysis of histone H3 protein acetylation mark on lysine residues K4, K9, K14, K18, K23, K27 and K56, in cells treated for 16 hours with 5 µM TSA, and challenged with the *E. coli* K12 strain. After cell lysis at the indicated time points, western-blots were performed using specific antibodies directed against histone post-translational modification marks. Experiments were performed at a MOI of 10 bacteria per cell. N = 2 independent experiments. NC: non-challenged cells ; K12: cells challenged with the *E. coli* K12 strain. “Ac” prefix: acetylation.

Figures 6. Implication of NF-κB in expression of the DEFB2 and IL8 genes upon inhibition of histone deacetylases. (A) Transcriptional expression of the DEFB2 and IL8 genes in cells transfected with siRNA against the p65 NF-κB subunit, treated or non-treated for 16 hours with 5 µM TSA, and challenged for 2 hours with the *E. coli* K12 strain. After mRNA extraction and RT reactions, qRT-PCR was performed on each sample with specific primers to determine the relative expression of genes using the comparative Ct method. Values are presented on a logarithmic scale as the ratio of gene expression in challenged cells, treated or non-treated, compared to non-challenged and non-treated cells. Experiments were performed at MOI of 10 bacteria per cell. N = 3 independent experiments. Error bars represent the SD. *, $P < 0,01$ for cells transfected with p65 siRNA, compared to cells transfected with scramble siRNA. Black bars: p65 siRNA transfected cells ; white bars: scramble siRNA transfected cells (SC). **(B)** Kinetic of expression of the DEFB2 and IL8 genes in cells inhibited for the NF-κB pathway by treatment with the BMS-345541 IKK inhibitor, treated or non-treated for 16 hours with 5 µM TSA, and challenged with the *E. coli* K12 strain. After mRNA extraction and RT reactions, qRT-PCR was performed on each sample with specific primers to determine the relative expression of genes using the comparative Ct method. Values are presented on a logarithmic scale for NF-κB inhibited cells

(dashed lines), and NF-κB non-inhibited cells (solid lines), as the ratio of gene expression in treated and challenged cells (black lines), or non-treated and challenged cells (grey lines), compared to non-treated and non-challenged cells. Experiments were performed at a MOI of 10 bacteria per cell. N = 3 independent experiments. Error bars represent the SD.

Figures 7. Acetylation of the NF-κB p65 subunit on serine residue K310 upon inhibition of histone deacetylases. Immunoblot analysis of the p65, p50, and its precursor p105 subunit proteins of the NF-κB transcription factor complex, the IκBα sequestration protein, and the acetylation mark of the p65 subunit on serine residue K310 (Ac-p65), in cells treated for 16 hours with 5 µM TSA, and challenged with the *E. coli* K12 strain. After cell lysis at the indicated time points, western-blots were performed using specific antibodies directed against proteins or post-translational modification marks. Experiments were performed at a MOI of 10 bacteria per cell. N = 2 independent experiments. NC: non-challenged cells ; K12: cells challenged with the *E. coli* K12 strain. “Ac” prefix: acetylation.

Figures 8. Implication of p300 in acetylation of NF-κB and induction of the DEFB2 gene expression. (A) Immunoblot analysis of the p300 histone acetyltranferase protein, and the acetylation mark of the p65 subunit on serine residue K310 (Ac-p65), in cells transfected with siRNA against the histone acetyltransferase p300, and challenged for 1 hours with the *E. coli* K12 strain. After cell lysis at the indicated time points, wester-blots were performed using specific antibodies directed against proteins or post-translational modification marks. Experiments were performed at a MOI of 10 bacteria per cell. N = 2 independent experiments. NC: non-challenged cells ; K12: cells challenged with the *E. coli* K12 strain ; SC: scramble siRNA transfected cells ; p300: p300 siRNA transfected cells. “Ac” prefix: acetylation. **(B)** Transcriptional expression of the DEFB2 and IL8 genes in cells transfected with siRNA directed against the histone acetyltransferase p300, and challenged for 2 hours with the *E. coli* K12 strain. After mRNA extraction and RT reactions, qRT-PCR was performed on each sample with specific primers to determine the relative expression of genes using the comparative Ct method. Values

are presented on a logarithmic scale as the ratio of gene expression in challenged cells compared to non-challenged cells. Experiments were performed at MOI of 10 bacteria per cell. N = 3 independent experiments. Error bars represent the SD. *, P < 0,01 for cells transfected with p300 siRNA, compared to cells transfected with scramble siRNA. Black bars: p300 siRNA transfected cells ; white bars: scramble siRNA transfected cells (SC). **(C)** Transcriptional expression of the DEFB2 and IL8 genes in cells treated with increasing concentrations of the p300 inhibitor C646 (0-50 µM C646), and challenged for 2 hours with the *E. coli* K12 strain. After mRNA extraction and RT reactions, qRT-PCR was performed on each sample with specific primers to determine the relative expression of genes using the comparative Ct method. Values are presented on a logarithmic scale, as the ratio of gene expression in challenged cells, compared to non-challenged cells. Experiments were performed at a MOI of 10 bacteria per cell. N = 3 independent experiments. Error bars represent the SD. *, P < 0,01 for cells treated with C646, compared to non-treated cells.

Figure 9. Transcriptomic analysis of intestinal epithelial cells upon inhibition of histone deacetylases. (A) Transcriptional expression of the whole genome of cells treated or non-treated for 16 hours with 5 µM TSA, and challenged for 1 hour with the *E. coli* K12 strain. After mRNA extraction, RNA sequencing was performed on each sample to determine the number of read per gene. Values are presented on a logarithmic scale as the ratio of gene expression in challenged cells compared to non-challenged cells (X axis) *versus* gene expression in treated and challenged cells compared to non-treated and non-challenged cells (Y axis). Experiments were performed at a MOI of 10 bacteria per cell. **(B-C)** Focus on the transcriptional expression of genes from the antimicrobial defence and epithelial restitution clusters **(B)**, and the transcriptional expression of genes from the pro-inflammatory pathway cluster **(C)**. Values are presented on a logarithmic scale as the ratio of gene expression in challenged cells compared to non-challenged cells (white bars), and treated and challenged cells compared to non-treated and non-challenged cells (black bars).

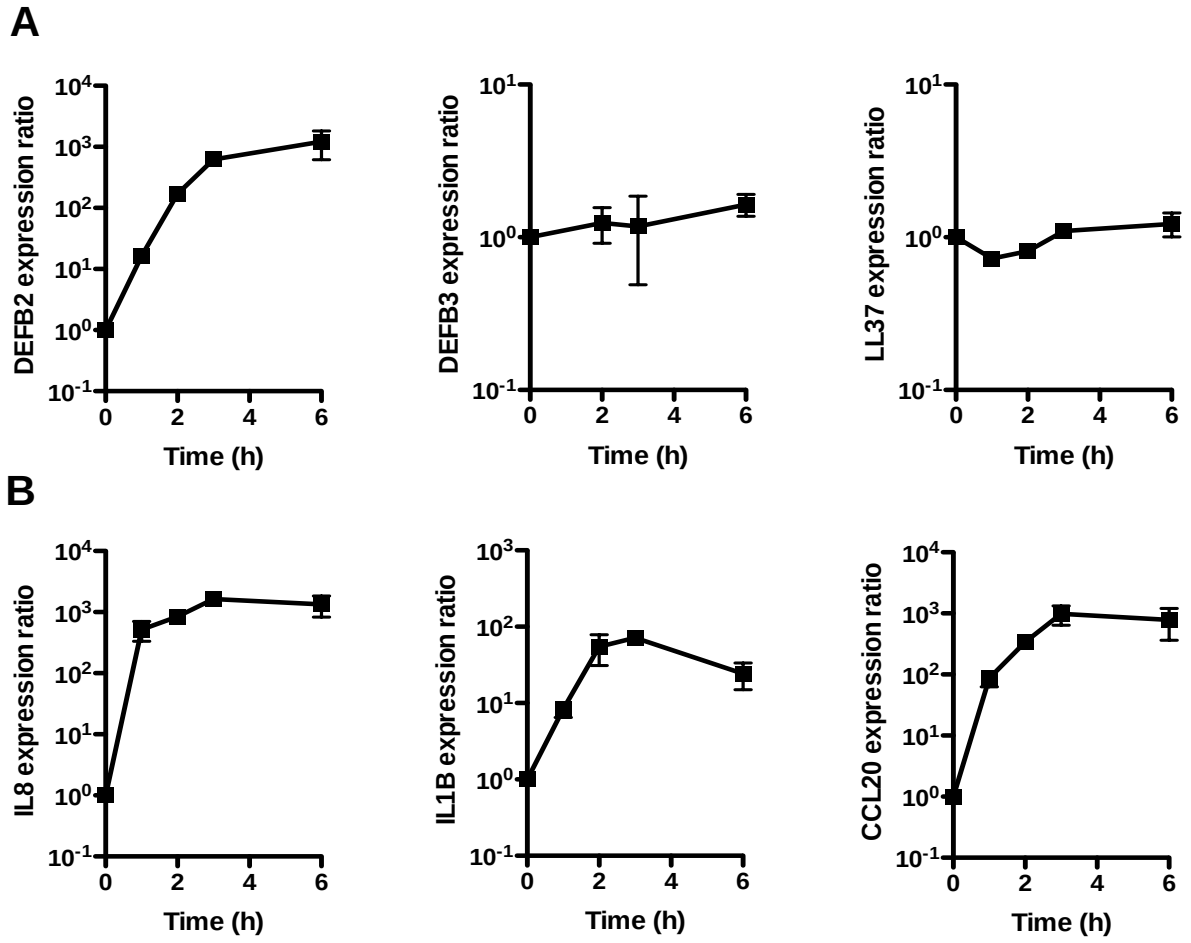
Supplemental figure 1. Expression of antimicrobial peptides and pro-inflammatory mediators upon inhibition of histone deacetylases by suberoylanilide hydroxamic acid. (A) Transcriptional expression of the DEFB2, DEFB3, and LL37 antimicrobial genes, and IL1B, IL8, and CCL20 pro-inflammatory genes, in cells treated for 16 hours with increasing concentrations of suberoylanilide hydroxamic acid (0-500 µM SAHA), and challenged for 2 hours with the *E. coli* K12 strain. After mRNA extraction and RT reactions, qRT-PCR was performed on each sample with specific primers to determine the relative expression of genes using the comparative Ct method. Values are presented on a logarithmic scale as the ratio of gene expression in treated and challenged cells (black bars), or treated and non-challenged cells (white bars), compared to non-treated and non-challenged cells. Experiments were performed at a MOI of 10 bacteria per cell. N = 3 independent experiments. Error bars represent the SD. *, P < 0,01 for treated cells, challenged or not, compared to non-treated and non-challenged cells. **(B)** ELISA measurement of the amount of DEFB2 and IL8 proteins detected in supernatants of cells treated or non-treated for 16 hours with 5 µM SAHA, and challenged or non-challenged for 6 hours with the *E. coli* K12 strain. Bacterial challenges were stopped at 6 hours by addition of gentamicin, and supernatants were collected 24 hours after the beginning of the challenge. Experiments were performed at a MOI of 10 bacteria per cell. Values are presented on a logarithmic scale in picogram of protein per milliliter. N = 3 independent experiments. NC: non-challenged cells ; K12: cells challenged with the *E. coli* K12 strain. Black bars: 5 µM SAHA treated cells ; grey bars: non-treated cells. Error bars represent the SD. *, P < 0,01 for treated cells compared to non-treated cells. **(C)** Cellular viability of cells treated for 16 hours with increasing concentrations of suberoylanilide hydroxamic acid (0-500 µM SAHA), and challenged for 2 hours with *E. coli* K12. Lactate dehydrogenase assays were performed with the supernatant of treated and challenged cells. Values represent the percentage of cell death. N = 3 independent experiments. Error bars represent the SD.

Supplemental figure 2. Acetylation of histone H4 lysine residues upon inhibition of histone deacetylases. Immunoblot analysis of histone H4 protein acetylation mark on lysine residues K5, K8, K12 and K16, in cells treated for 16 hours with 5 µM TSA, and challenged with the *E. coli* K12 strain. After cell lysis at the

indicated time points, western-blots were performed using specific antibodies directed against histone post-translational modification marks. Experiments were performed at a MOI of 10 bacteria per cell. N = 2 independent experiments. NC: non-challenged cells ; K12: cells challenged with the *E. coli* K12 strain. “Ac” prefix: acetylation.

Figures

Figure 1:



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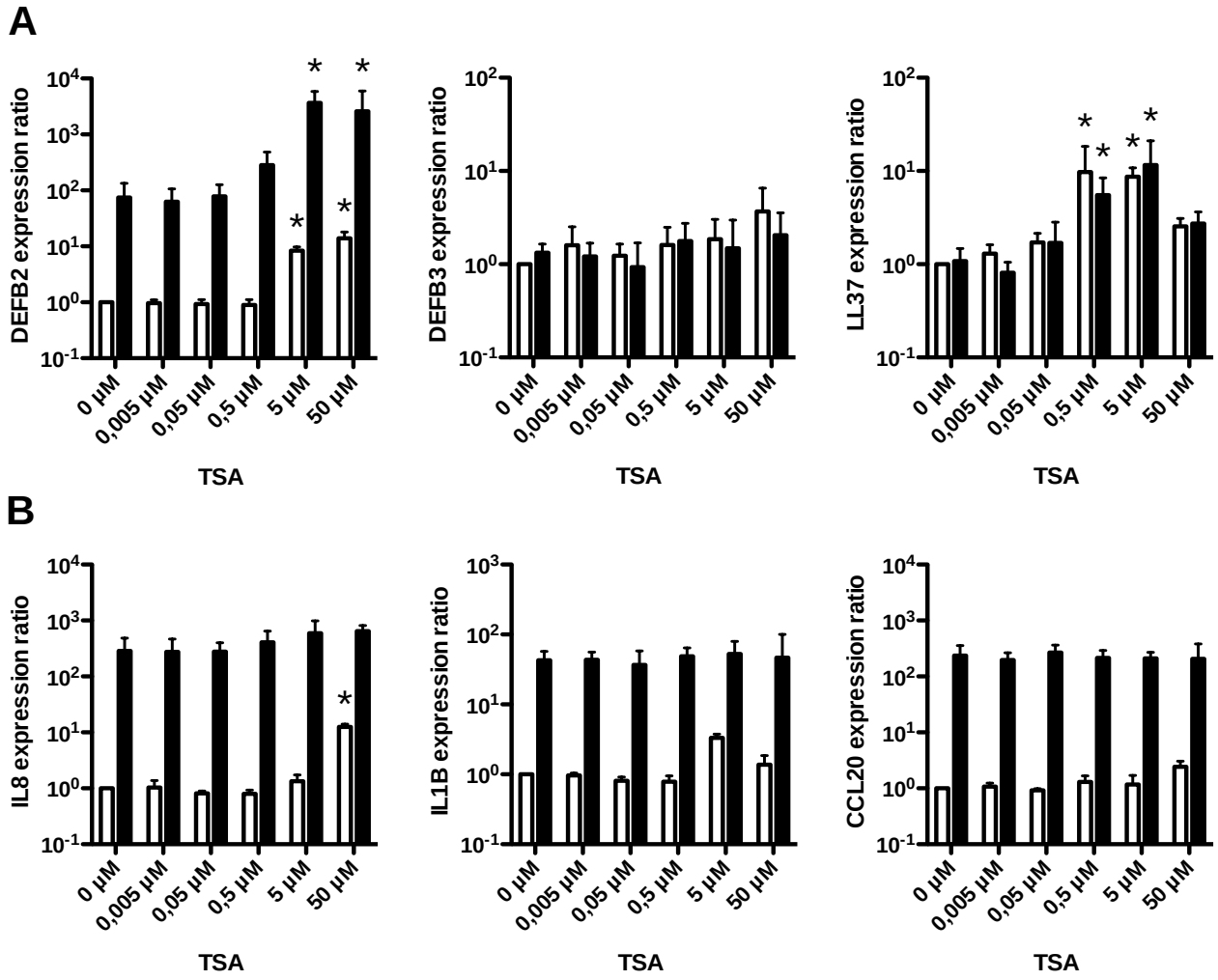
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DEFB2 expression ratio
DEFB3 expression ratio
LL37 expression ratio
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5 µM
50 µM
TSA
B
IL8 expression ratio
IL1B expression ratio
CCL20 expression ratio

Figure 3:

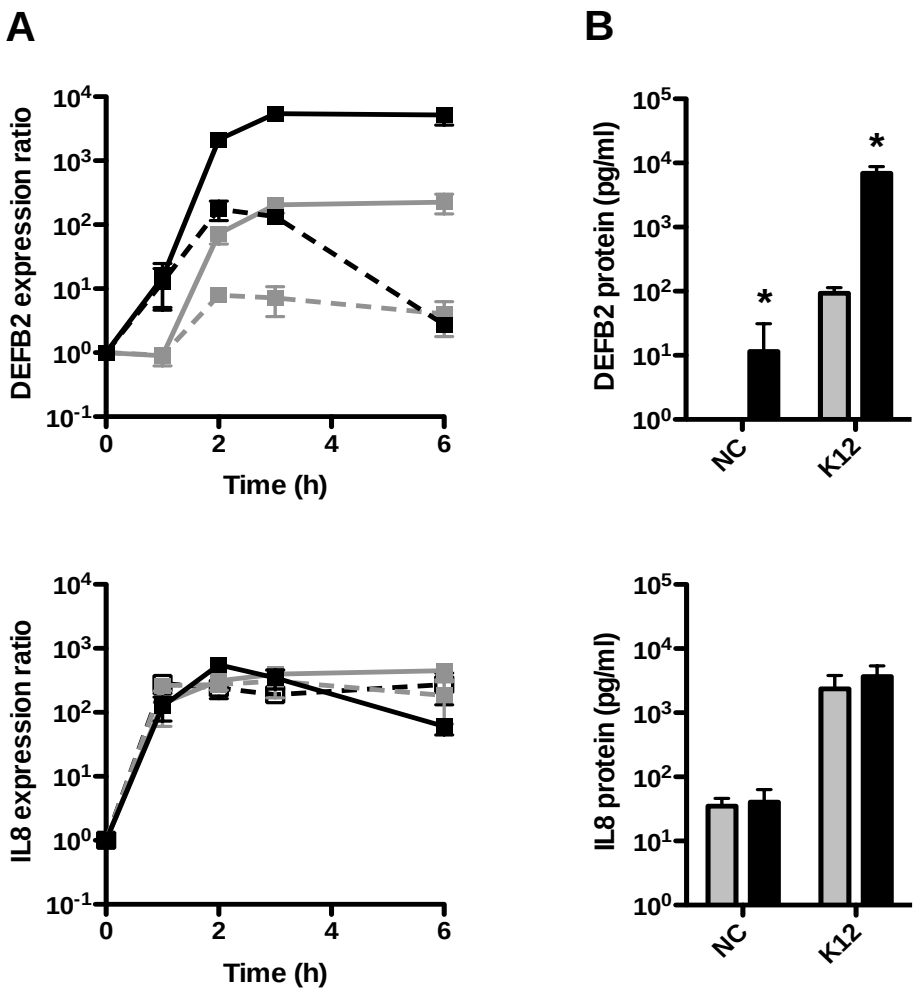

A
B
DEFB2 expression ratio
Time (h)
DEFB2 protein (pg/ml)
NC
K12
IL8 expression ratio
Time (h)
IL8 protein (pg/ml)
NC
K12

Figure 4:

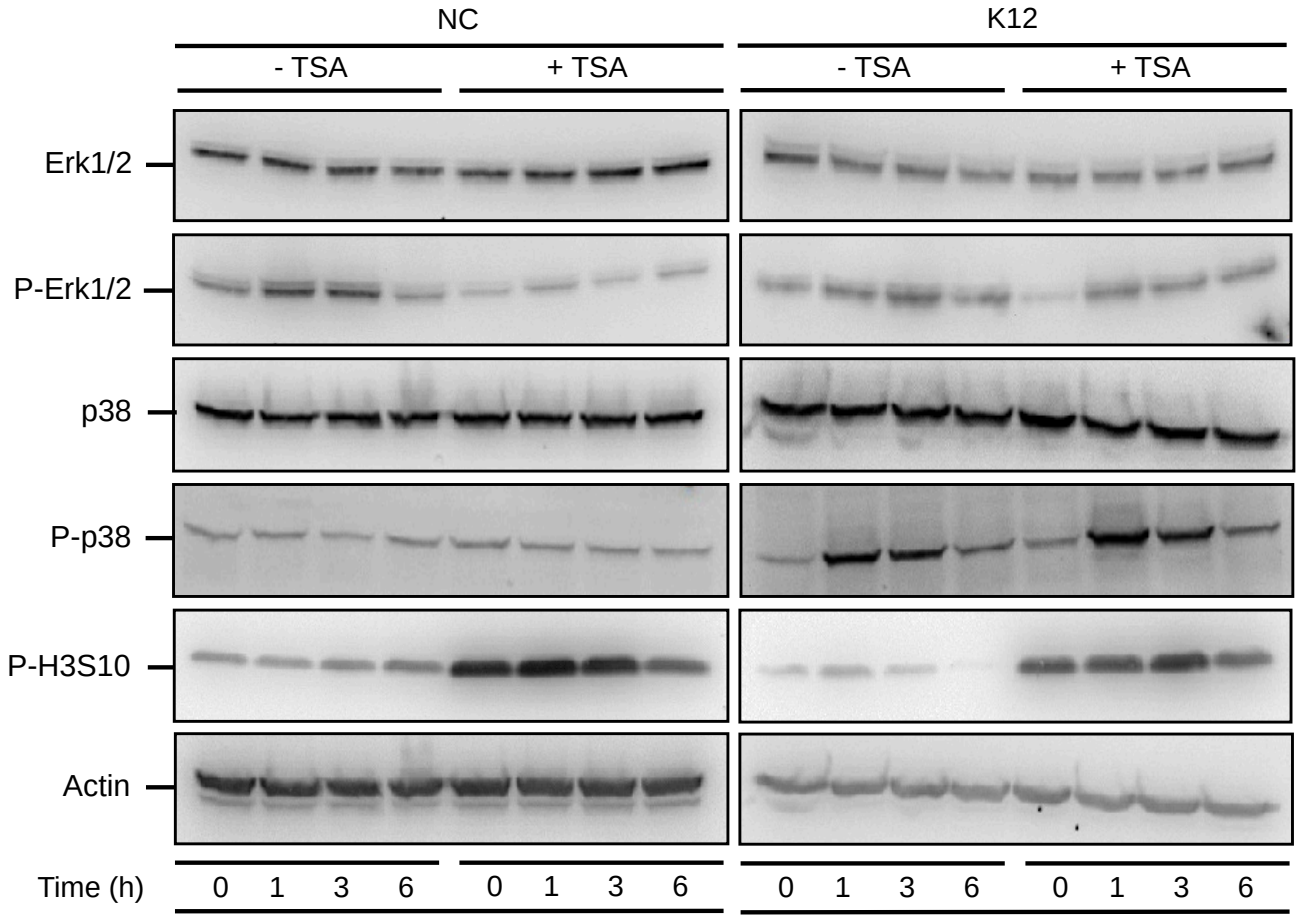

NC
K12
- TSA
+ TSA
- TSA
+ TSA
Erk1/2
P-Erk1/2
p38
P-p38
P-H3S10
Actin
Time (h)
0 1 3 6 0 1 3 6
0 1 3 6 0 1 3 6

Figure 5:

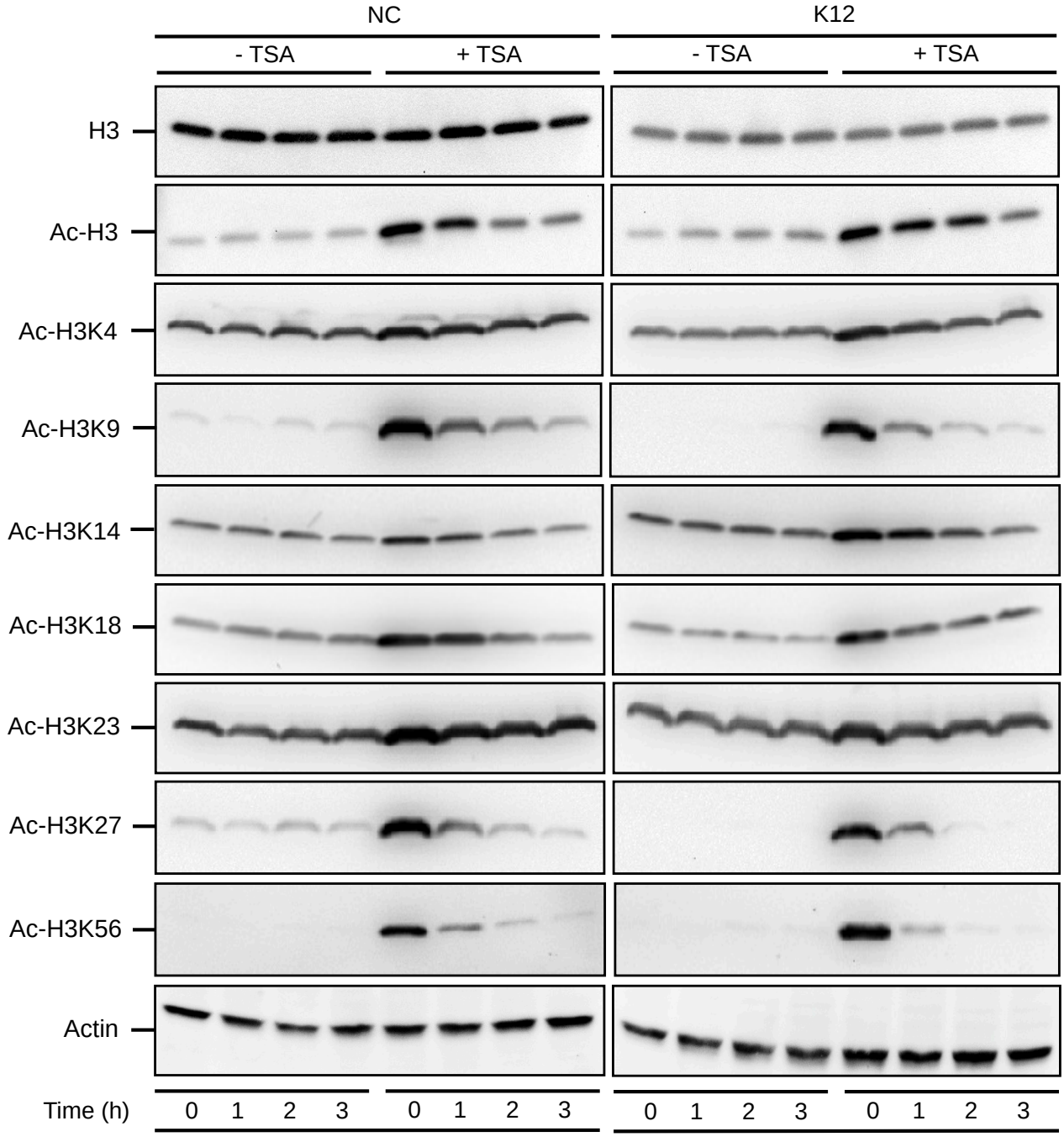

NC
K12
- TSA
+ TSA
- TSA
+ TSA
H3
Ac-H3
Ac-H3K4
Ac-H3K9
Ac-H3K14
Ac-H3K18
Ac-H3K23
Ac-H3K27
Ac-H3K56
Actin
Time (h)
0 1 2 3 0 1 2 3
0 1 2 3 0 1 2 3

Figure 6:

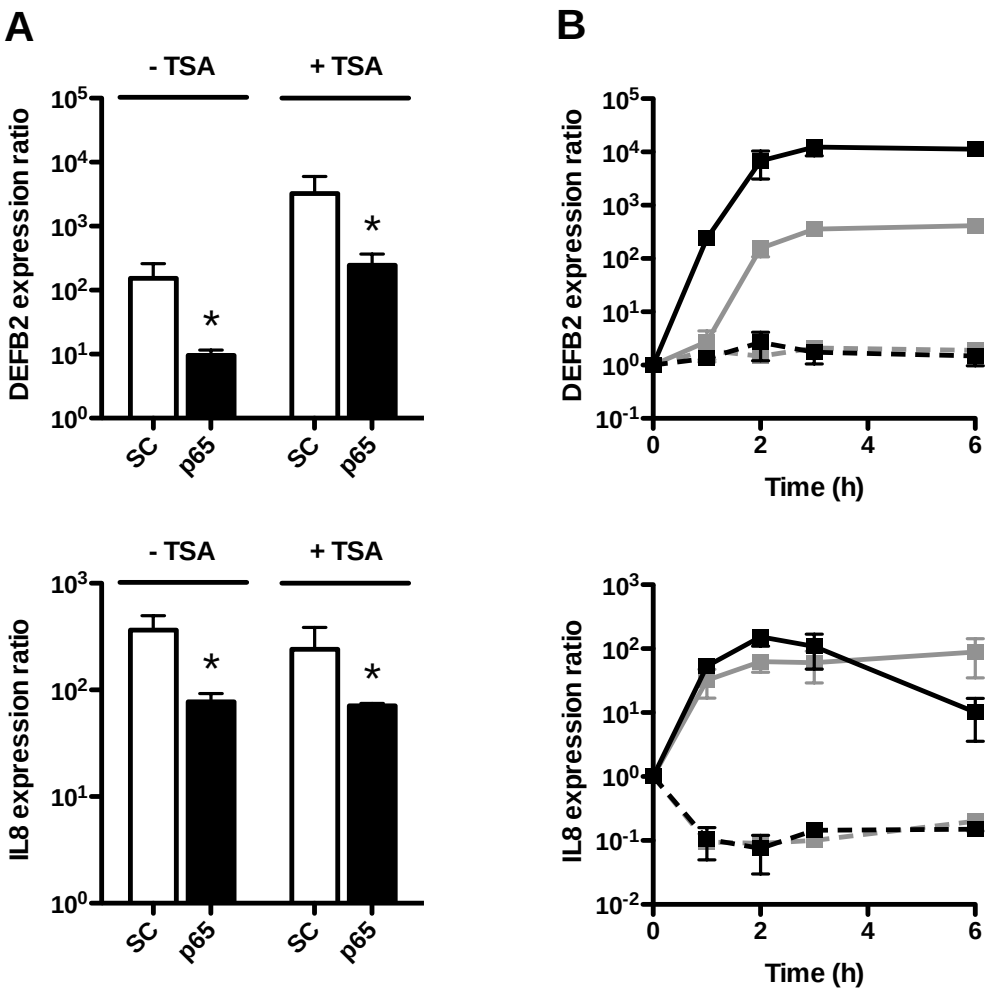
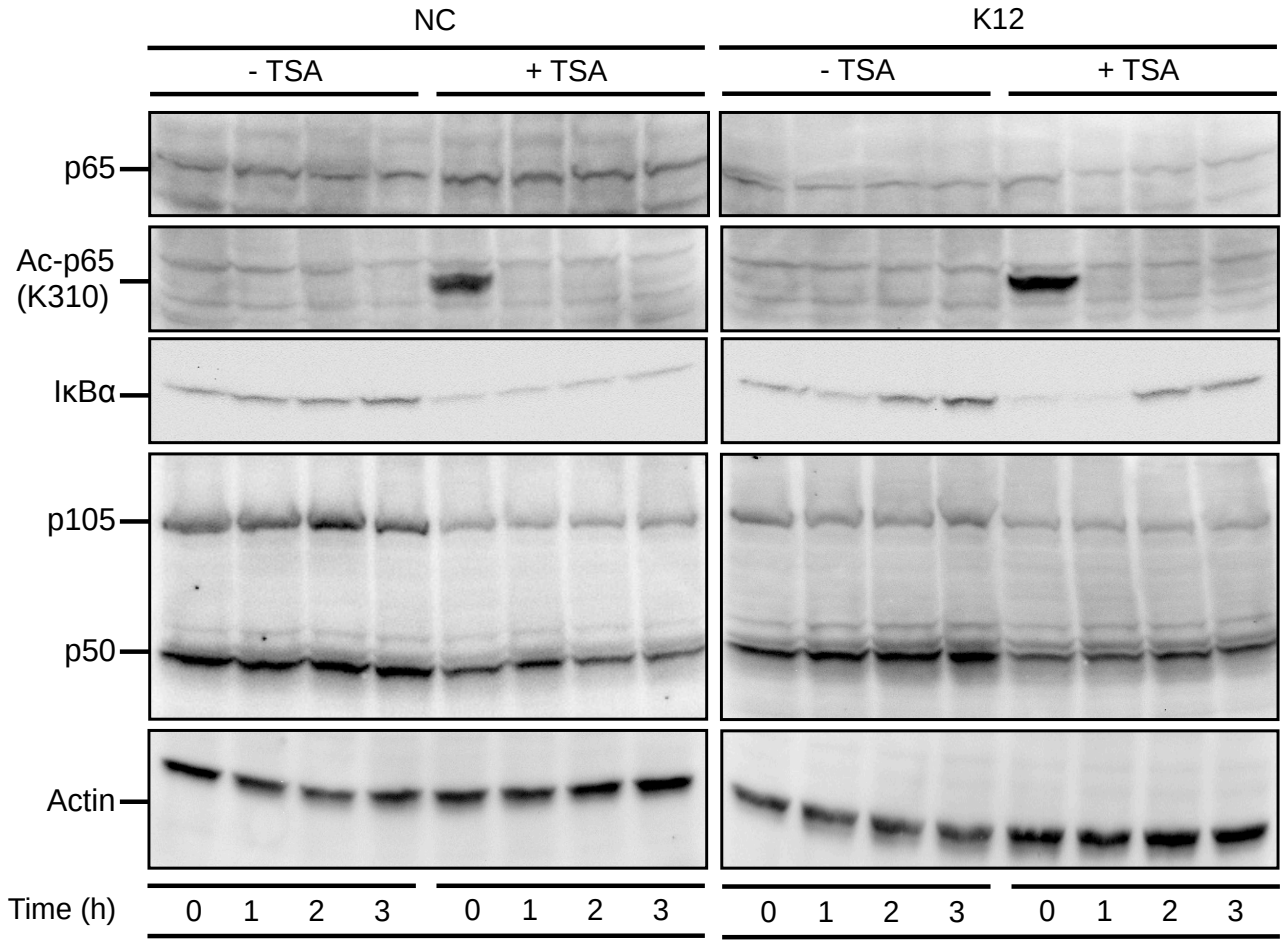

A
- TSA
+ TSA
DEFB2 expression ratio
SC
p65
SC
p65
IL8 expression ratio
B
DEFB2 expression ratio
Time (h)
IL8 expression ratio
Time (h)

Figure 7: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K12
- TSA
+ TSA
- TSA
+ TSA
p65
Ac-p65
(K310)
IκBα
p105
p50
Actin
Time (h)
0 1 2 3 0 1 2 3
0 1 2 3 0 1 2 3

Figure 8:

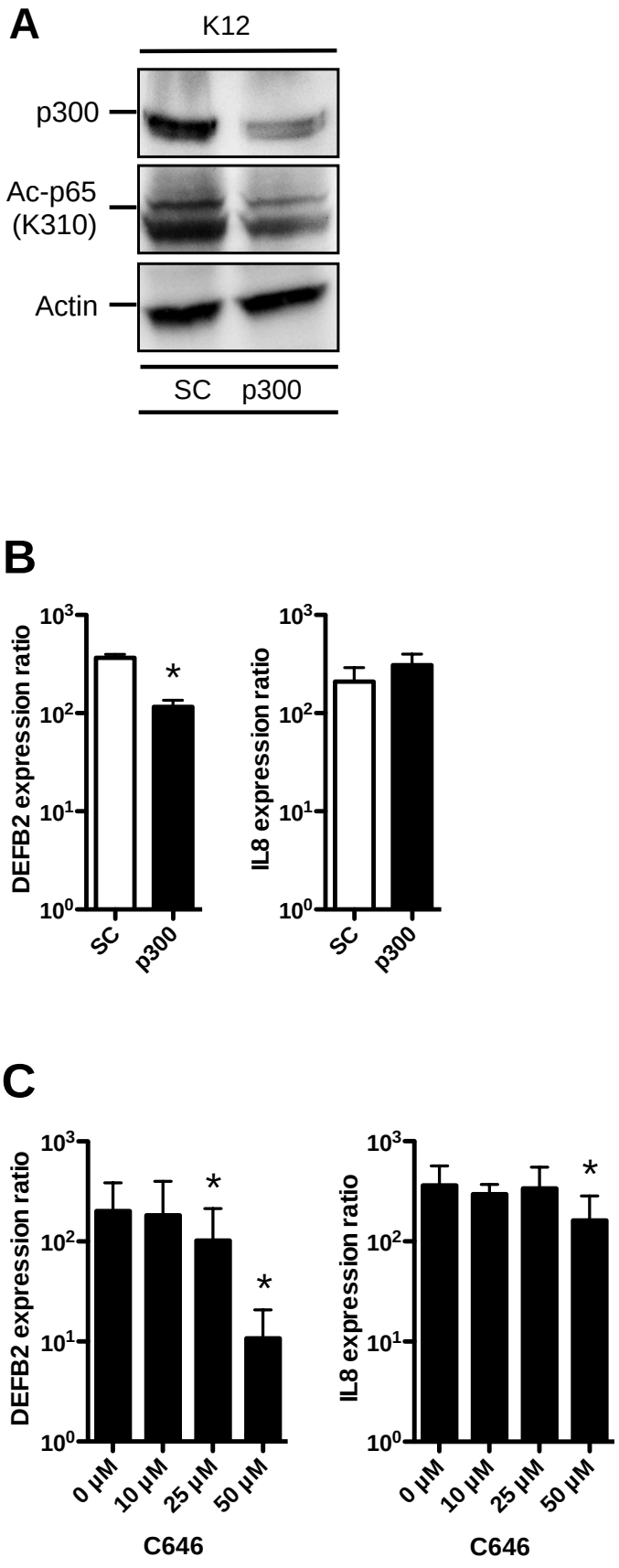
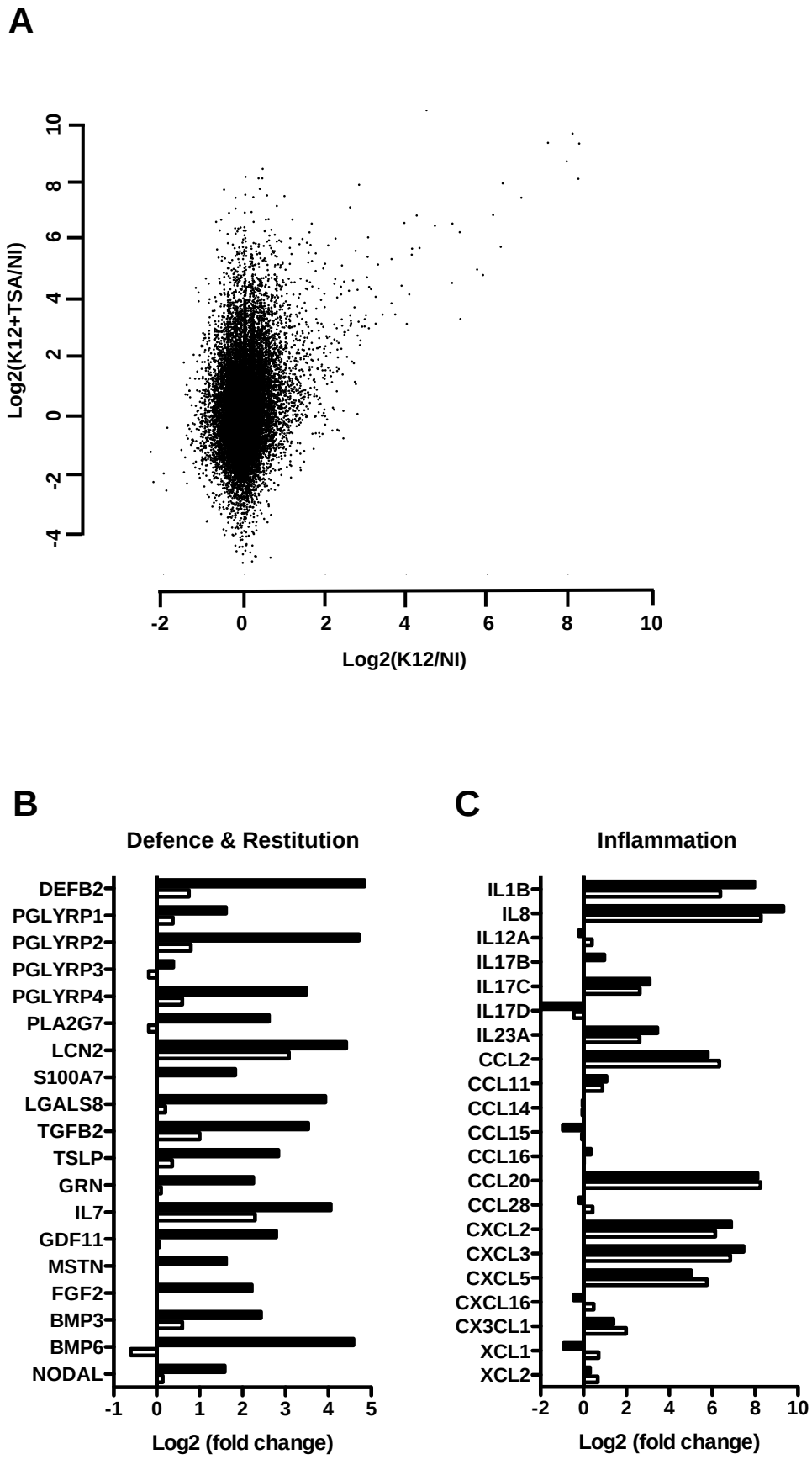

A
K12
p300
Ac-p65
(K310)
Actin
SC
p300
B
DEFB2 expression ratio
IL8 expression ratio
SC
p300
C
DEFB2 expression ratio
IL8 expression ratio
0 µM
10 µM
25 µM
50 µM
C646

Figure 9:


A
Log2(K12+TSA/NI)
10
8
6
4
2
0
-2
-4
-2
0
2
4
6
8
10
Log2(K12/NI)
B
Defence & Restitution
DEFB2
PGLYRP1
PGLYRP2
PGLYRP3
PGLYRP4
PLA2G7
LCN2
S100A7
LGALS8
TGFB2
TSLP
GRN
IL7
GDF11
MSTN
FGF2
BMP3
BMP6
NODAL
-1
0
1
2
3
4
5
Log2 (fold change)
C
Inflammation
IL1B
IL8
IL12A
IL17B
IL17C
IL17D
IL23A
CCL2
CCL11
CCL14
CCL15
CCL16
CCL20
CCL28
CXCL2
CXCL3
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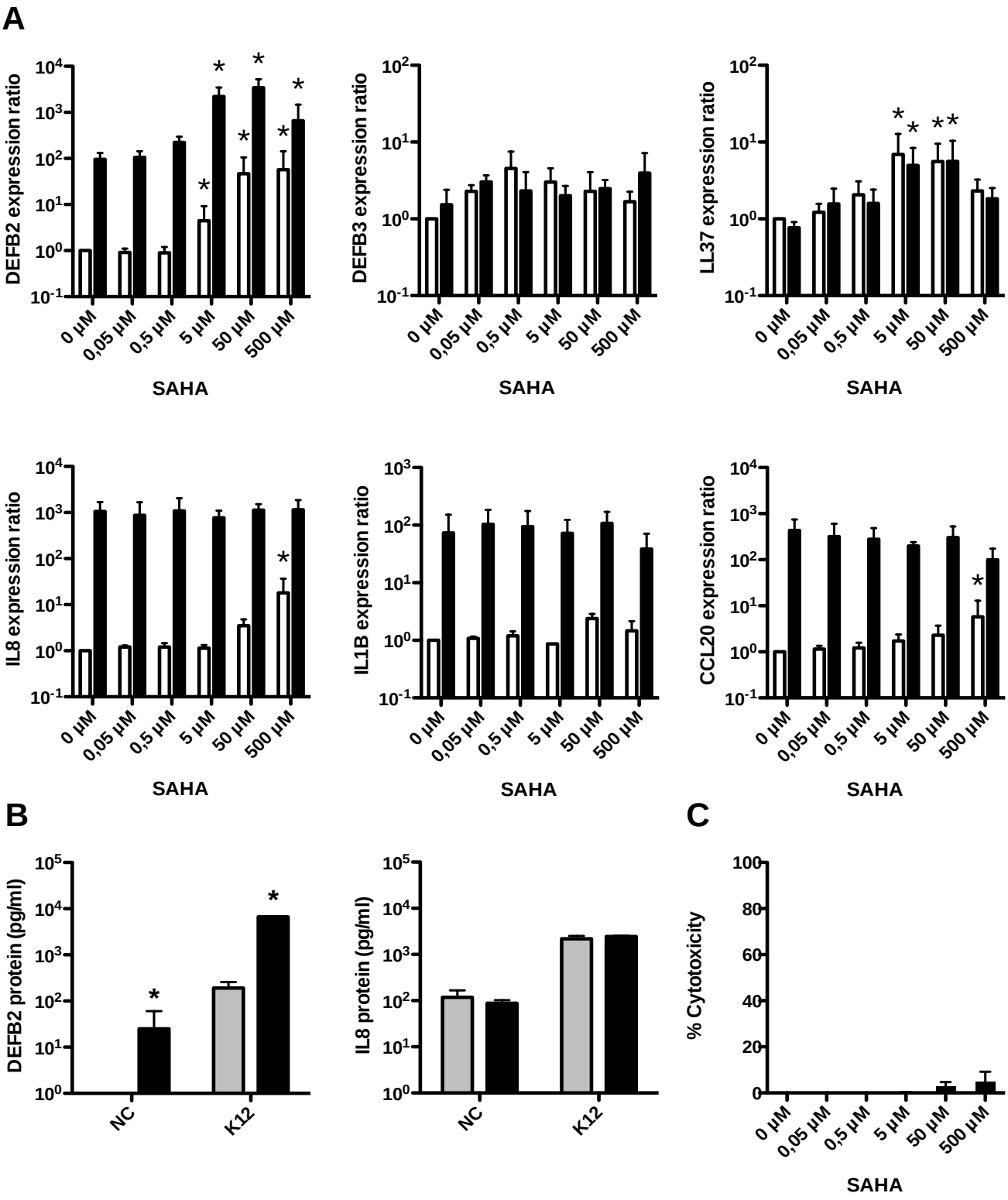
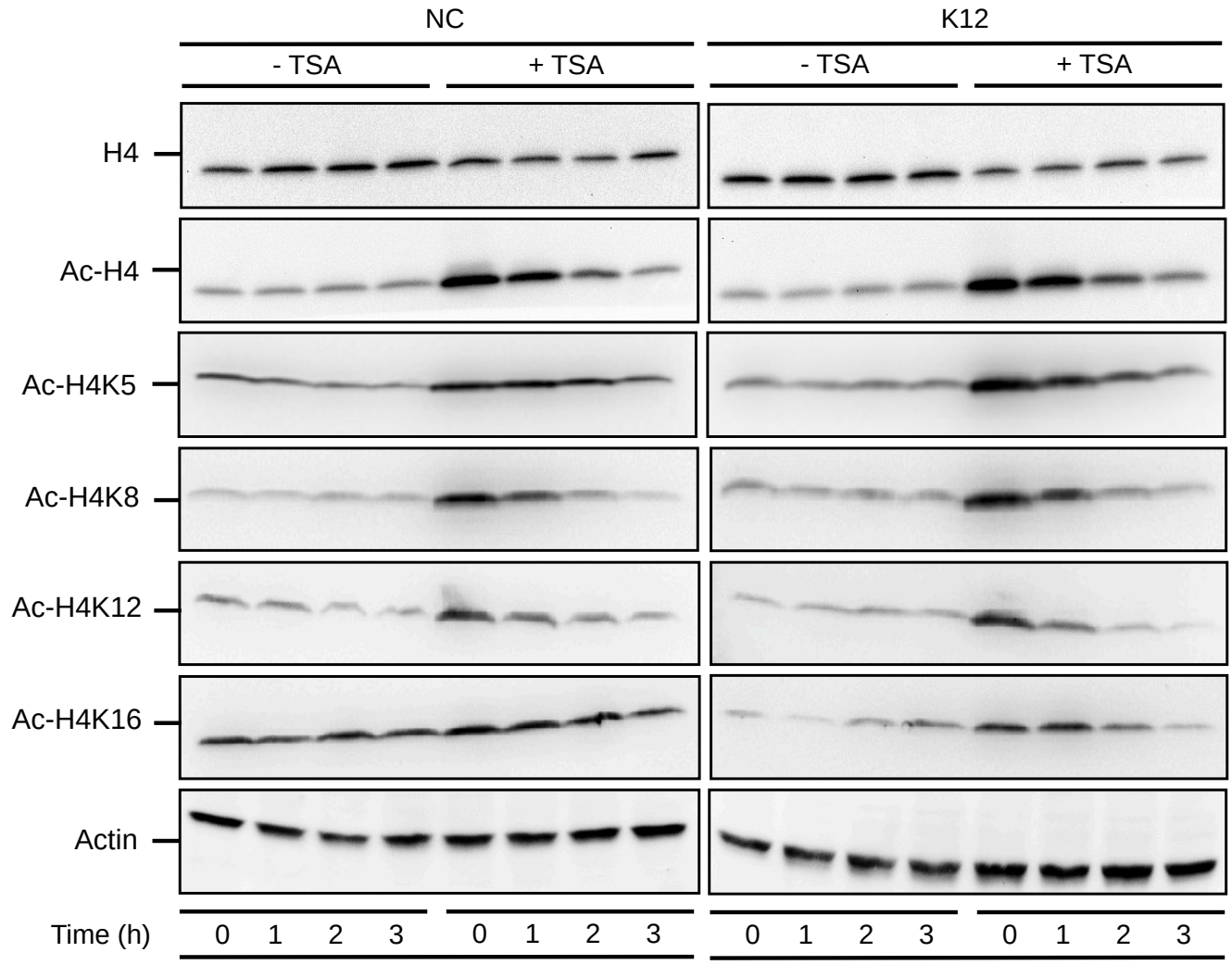
-2
0
2
4
6
8
10
Log2 (fold change)

Supplementary Figure 1:



Supplementary Figure 2:



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allosteric site of the enzyme and blocks NF-kappa B-dependent transcription in mice. *J. Biol. Chem.* 278: 1450–1456.

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V.2 Additional Results

This chapter is divided into two parts: The first part concerns results obtained with the *E. coli* strain K12, in addition to the results presented in the manuscript in preparation (V.2.1). The second part contains results obtained with an additional bacterial strain, *E. coli* LF82 (V.2.2). The additional results section will be followed by a short section of additional methods (V.3)

V.2.1 Additional results for challenge with the *E. coli* strain K12

The previously presented manuscript in preparation contains the most striking results concerning the expression and regulation of the DEFB2 and IL8 genes upon challenge of intestinal epithelial cells with the *E. coli* strain K12. In this section those results are completed with observations concerning the knockdown of HDAC (V.2.1.1) and HAT (V.2.1.2) enzymes prior to bacterial challenge.

V.2.1.1 Knockdown of histone deacetylases differentially influences the expression of DEFB2 and IL8 genes upon E. coli K12 challenge

As we observed that the inhibition of the classical histone deacetylases by the use of the pan-inhibitor TSA resulted in the strong increase of expression of AMP genes upon bacterial challenge, we wanted to investigate the involvement of single HDAC enzymes or HDAC classes in this process. Therefore, intestinal epithelial cells were transfected with siRNAs targeting either single HDAC enzymes, a combination to target a single class or a combination of several classes. After knockdown for 48h, cells were challenged with the *E. coli* strain K12 for 2h and the expression of the DEFB2 and IL8 genes was evaluated using qRT-PCR. Interestingly, we did not observe an increased DEFB2 expression upon knockdown of a single HDAC enzyme, not a single HDAC class (Figure A1 A, upper panel), nor knockdown of combination of classes, or all classical HDAC enzymes together (Figure A1 B, upper panel). Moreover, knockdown of some HDAC enzymes significantly decrease the expression of DEFB2 upon *E. coli* challenge, with the exception of single knockdown of HDAC1 and most of HDACs of class IIa. On the contrary, knockdown of most single HDAC enzymes significantly increased the expression of IL8 upon *E. coli*

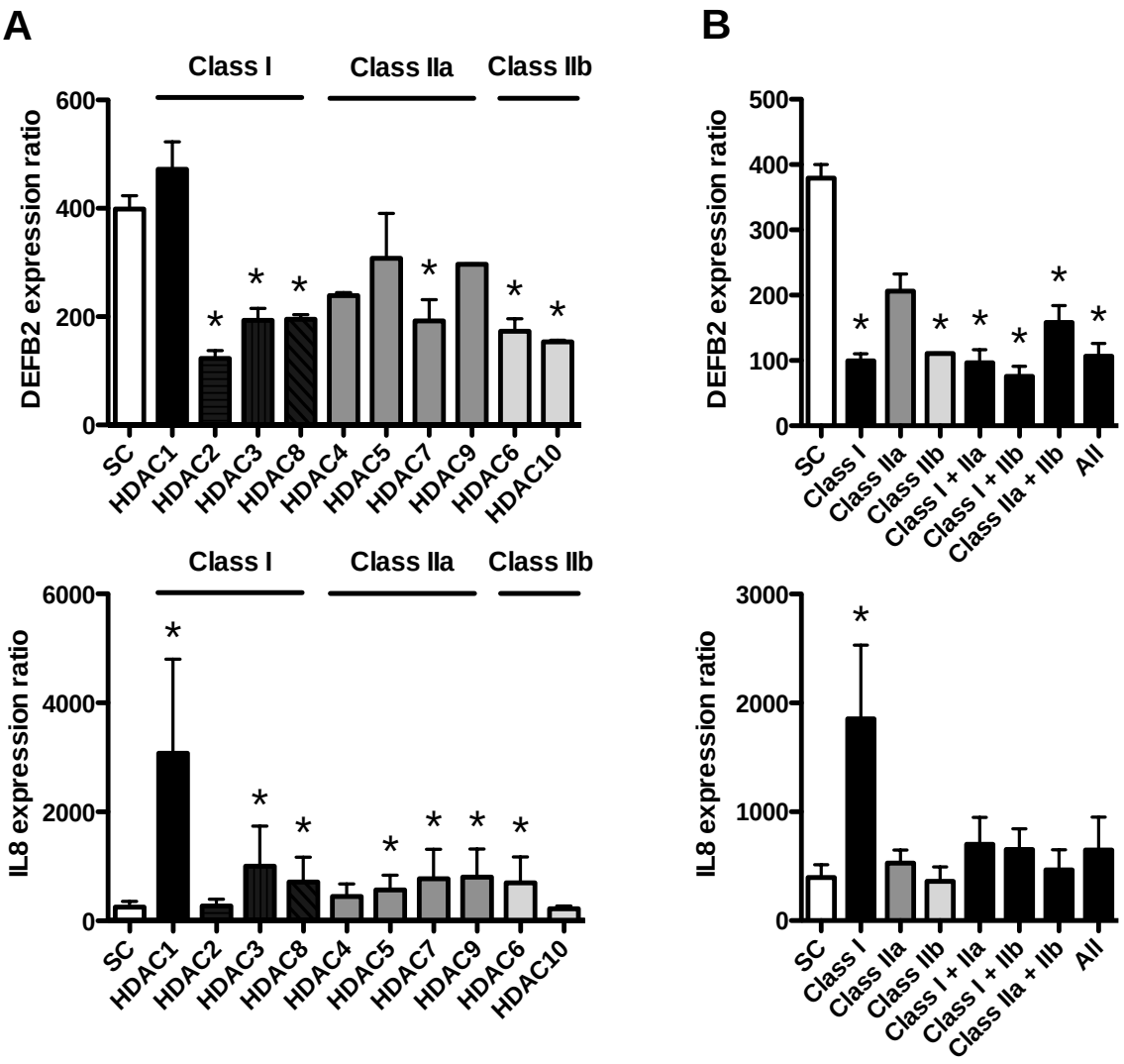


Figure A1: Influence of HDAC enzyme knockdown on the expression of DEFB2 and IL8 upon challenge with *E. coli* K12. Transcriptional expression of the DEFB2 and IL8 genes in cells transfected with siRNA directed against single histone deacetylases **(A)** or in a combination to target groups of histone deacetylases **(B)**, and challenged for 3 hours with the *E. coli* K12 strain. After mRNA extraction and RT reactions, qRT-PCR was performed on each sample with specific primers to determine the relative expression of genes using the comparative Ct method. Values are presented on a logarithmic scale as the ratio of gene expression in challenged cells compared to non-challenged cells. Experiments were performed at MOI of 10 bacteria per cell. N = 3 independent experiments. Error bars represent the SD. *, P < 0,01 for cells transfected with HDAC targeting siRNA, compared to cells transfected with scramble siRNA. Black bars: HDAC class I siRNA transfected cells; dark grey bars: HDAC class II siRNA transfected cells; light grey bars: HDAC class III siRNA transfected cells; white bars: scramble siRNA transfected cells (SC).

challenge, especially HDAC1 (Figure A1 A, lower panel). Interestingly, this effect is lost in the knockdown of HDAC classes, except class I, or combination of HDAC classes (Figure A1 B, lower panel). At this point we can conclude that the effect of increased expression of AMPs upon histone deacetylase inhibitor treatment is not mediated by a single HDAC and cannot be obtained upon knockdown of HDAC enzymes. As these enzymes play such versatile roles in the cell and act together not only with each other, but also with other proteins within protein complexes, it can be imagined the difficulty of deciphering their explicit role in gene expression. This will be discussed in more detail in chapter VI.

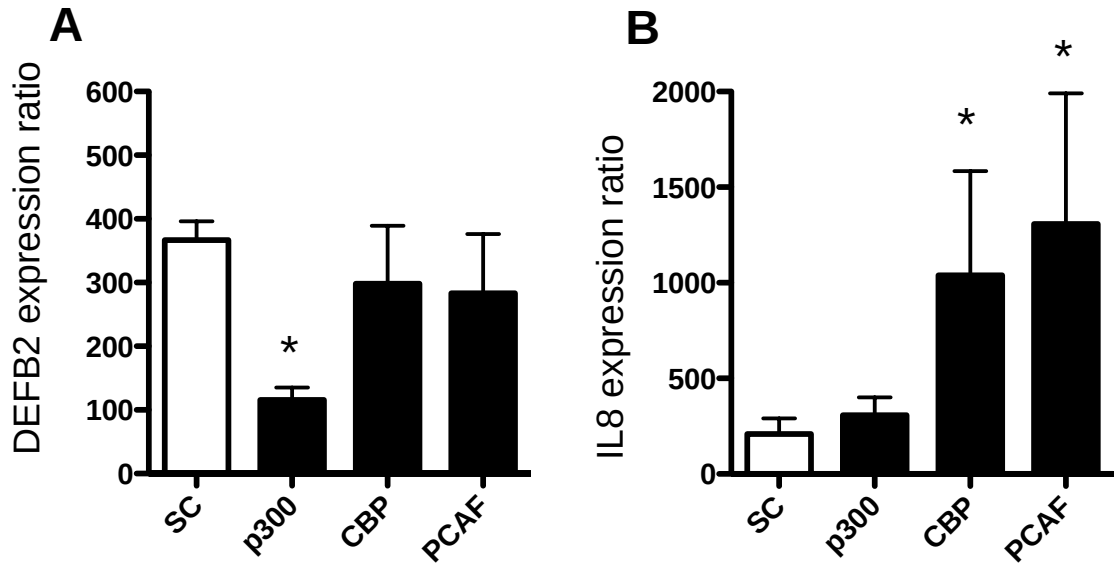


Figure A2: Influence of HAT enzyme knockdown on the expression of DEFB2 and IL8 upon challenge with *E. coli* K12. Transcriptional expression of the DEFB2 (**A**) and IL8 (**B**) genes in cells transfected with siRNA directed against single histone acetyltransferases, and challenged for 3 hours with the *E. coli* K12 strain. After mRNA extraction and RT reactions, qRT-PCR was performed on each sample with specific primers to determine the relative expression of genes using the comparative Ct method. Values are presented on a logarithmic scale as the ratio of gene expression in challenged cells compared to non-challenged cells. Experiments were performed at MOI of 10 bacteria per cell. N = 3 independent experiments. Error bars represent the SD. *, P < 0,01 for cells transfected with HAT targeting siRNA, compared to cells transfected with scramble siRNA. Black bars: HAT siRNA transfected cells; white bars: scramble siRNA transfected cells (SC).

V.2.1.2 Differential HATs are involved in the expression of DEFB2 and IL8

Since we discovered that acetylation seems to play a big role in the expression of AMPs, and we investigated already the role of HDAC enzymes, we wanted to know more about the involvement of different HAT enzymes. Therefore, we performed siRNA transfection experiments of intestinal epithelial cells to knockdown HAT enzymes belonging to different families, prior to challenge with *E. coli* strains. As already described in the presented manuscript, the knockdown of p300 had a significant effect on the bacteria-stimulated DEFB2 expression and reduced it significantly (Figure A2 A), while having no effect on the expression of IL8 (Figure A2 B). Knockdown of other HATs, such as CBP and PCAF, did not influence the induced DEFB2 expression (Figure A2 A). On the contrary, we observed a significant increase in bacteria-induced IL8 expression upon knockdown of CBP and PCAF (Figure A2 B). These results are interesting, as they present the involvement of different HAT enzymes in the expression of different gene, and moreover of two HATs which share such a high sequence homology as p300 and CBP. Furthermore, the data show a differential effect of different HATs in the stimulation or repression of expression of these genes, as knockdown can either lead to increased or decreased inducibility.

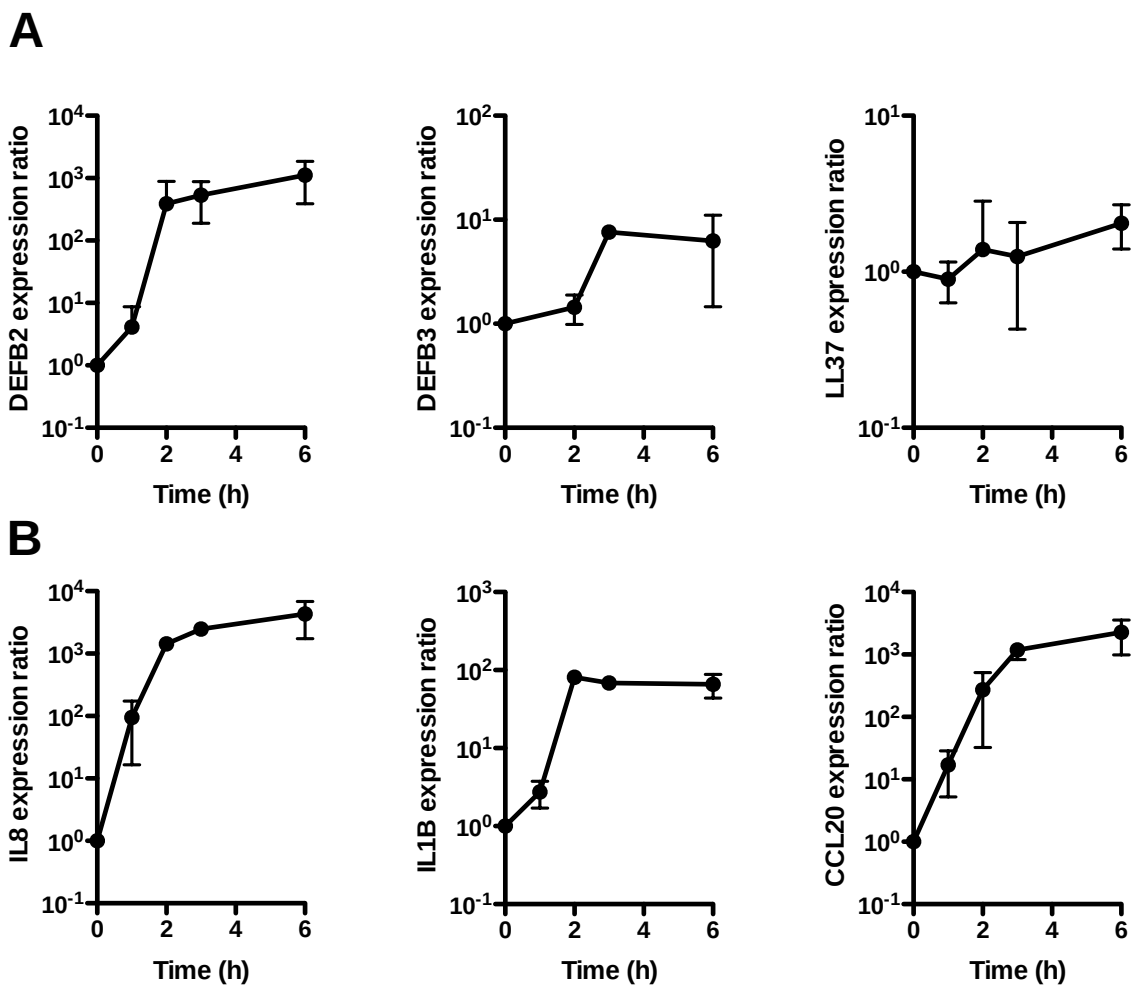


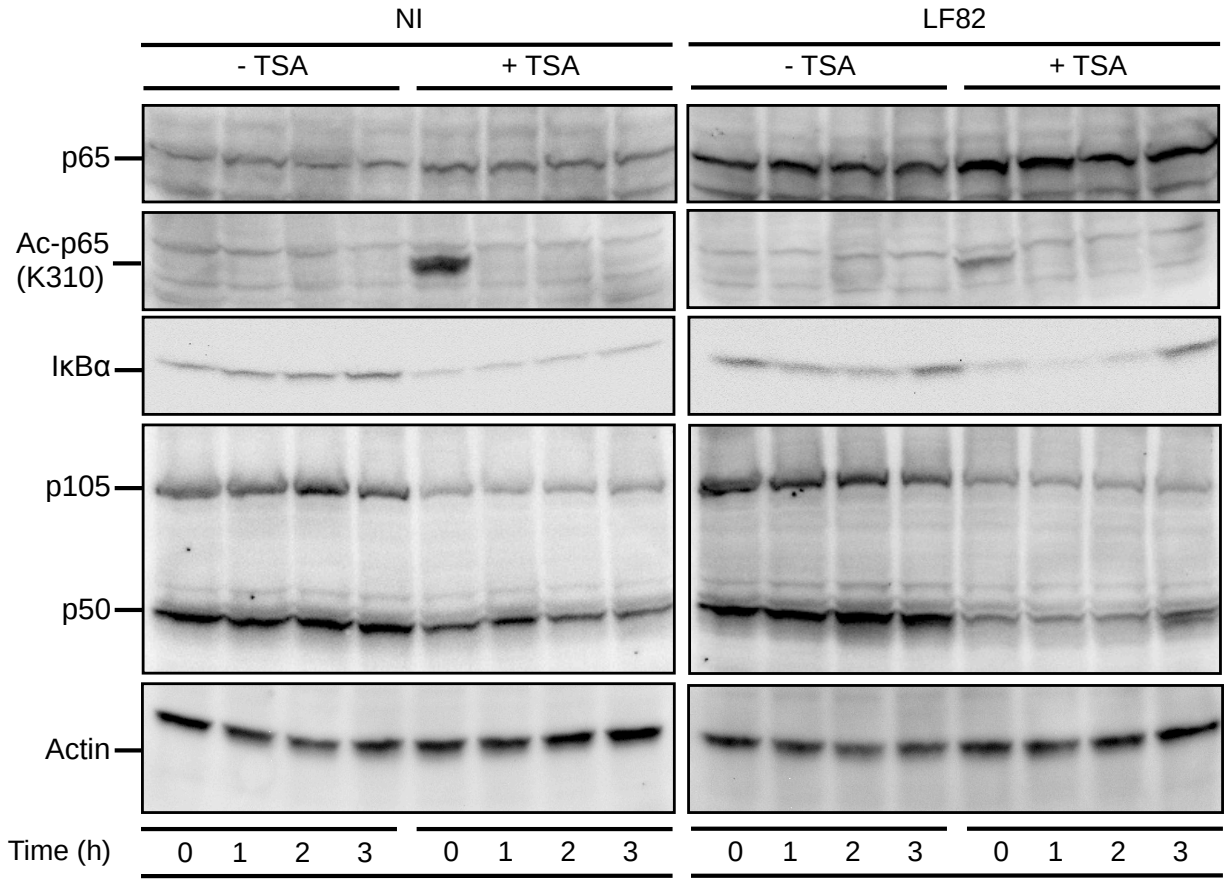
Figure A3: Expression of antimicrobial and pro-inflammatory genes upon challenge with *E. coli* LF82. Transcriptional expression of genes encoding the antimicrobial peptide beta-defensin DEFB2 and DEFB3, the cathelicidin LL37 **(A)**, and the pro-inflammatory mediators interleukins IL1B and IL8, and the chemokine CCL20 **(B)**, in cells challenged with the *Escherichia coli* LF82 strain. After mRNA extraction and RT reactions, qRT-PCR was performed on each sample, for each time point, with specific primers to determine the relative expression of genes using the comparative Ct method. Values are presented on a logarithmic scale as the ratio of gene expression in challenged cells compared to non-challenged cells. Experiments were performed at a MOI of 10 bacteria per cell. N = 3 independent experiments. Error bars represent the SD.

V.2.2 Additional results for challenge with the *E. coli* strain LF82

In the beginning of the thesis project we choose to work with two *E. coli* strains, in order to compare a commensal strain and a possible pathogenic strain. All experiments presented with the *E. coli* strain K12 were additionally performed in parallel with the *E. coli* strain LF82. This strain was isolated from a Crohn's disease patient [692] and is considered a pathobiontic strain, as it can adhere to, and invade cells and induces a strong TNFα response in macrophages [693]. We were surprised to not observe major differences in the expression or regulation of AMPs and INFs, with or without pre-treatment of epigenetic inhibitors, upon challenge of intestinal epithelial cells with these two strains throughout all presented experiments. Other results obtained in this comparative study were similar and are therefore not shown.The few differences we observed are presented in this section for challenge with the LF82 strain.

V.2.2.1 Challenge with E. coli stain LF82 shows differential kinetics of AMPs and INFs expression as compared to E. coli strain K12

For some of the examined AMPs and INFs genes we could observe differences in the kinetics and magnitude of expression after intestinal epithelial cell challenge with the *E. coli* strain LF82 as compared to K12. For the expression of DEFB2 we observe a slightly slower kinetic of expression within the first hour after challenge with LF82 as compared to K12, which then increases 2 log fold until 2h post challenge, to the same level as observed with K12 (Figure A3 A, left panel). The induction of DEFB3 expression, on the other hand, is significantly higher upon challenge with LF82 as compared to K12, especially after 3h post challenge (Figure A3 A, middle panel). For the expression of INFs genes, we observe a slower kinetic of induction for all three examined genes, within the first hour post challenge with LF82, as compared to K12 (Figure A3 B). Then, the level of INFs gene induction rises quickly to the same level as compared to K12 at 2h post challenge, and seems to even slightly exceed the level achieved with K12 challenge at 6h post challenge. These observations, concerning the slower kinetics of DEFB2, IL8, IL1β and CCL20 gene expression with LF82, especially within the first hour of challenge, could indicate mechanism applied by this pathobiontic strain, which allow for a restrain of the host response.



Figures A4: Acetylation of the NF-κB p65 subunit on serine residue K310 upon inhibition of histone deacetylases. Immunoblot analysis of the p65, p50, and its precursor p105 subunit proteins of the NF-κB transcription factor complex, the IκBα sequestration protein, and the acetylation mark of the p65 subunit on serine residue K310 (Ac-p65), in cells treated for 16 hours with 5 µM TSA, and challenged with the *E. coli* LF82 strain. After cell lysis at the indicated time points, western-blots were performed using specific antibodies directed against proteins or post-translational modification marks. Experiments were performed at a MOI of 10 bacteria per cell. N = 2 independent experiments. NC: non-challenged cells ; LF82: cells challenged with the *E. coli* LF82 strain. “Ac” prefix: acetylation.

V.2.2.2 LF82 shows prolonged activation of the NF-κB pathway

Besides the differences in kinetics and level of AMPs and INFs genes expression between the two *E. coli* strains, we observed a difference in the activation of NF-κB. The Western blot analysis of proteins participating in the NF-κB pathway after treatment of intestinal epithelial cells with histone deacetylate inhibitor and challenge with *E. coli* strains revealed some differences between challenge with the K12 and the LF82 strain. As already discussed the treatment with TSA decreased the protein level of the NF-κB inhibitor IκBα and the subunit p50/p105. Interestingly, the timing of re-synthesis of this proteins differed between K12 and LF82. After challenge with K12 the protein level of IκBα was observed back at basal level after 2h, while after challenge with LF82 it was only observed back at basal level after 3h post challenge (Figure A4). Additionally, challenge with LF82 after TSA pre-treatment, but not K12, lead to a continuing decreased level of the p50 protein and its precursor p105, which recovered after 3h post challenge. These observations indicate that the pathobiontic strain induces a longer activation of the NF-κB pathway and additionally influences NF-κB subunits differently as compared to the commensal K12. This could explain the higher and prolonged expression of INFs genes at later time points post challenge, as compared to K12 (Figure A3 B).

V.3 Additional Material and Methods

V.3.1 Bacterial strains

The *Escherichia coli* LF82 bacterial strain [694] was grown in LB medium (Sigma) at 37°C. For challenge experiments, cells were grown at confluence in 6-well plates (1.5×10^6 cells/well) for 48 h at 37°C and 10% CO_2. Bacterial challenges were performed using supplemented DME without antibiotics directly with over night bacterial cultures, at a multiplicity of infection (MOI) of 10 bacteria per cell, for indicated times. For experiments involving pharmacological inhibitors, cells were pretreated for 16 h over-night and washed with supplemented DME without antibiotics before infection.

V.3.2 siRNAs used for the knockdown of HDAC enzymes

The RNAi Max reagent (Invitrogen) was used to transfect cells with a final concentration of 25 nM siGENOME SMARTpool siRNAs (Thermo Scientific) targeting HDAC 1 (M-003493-02-0020), HDAC 2 (M-003495-02-0005), HDAC 3 (M-003496-02-0005), HDAC 4 (M-003497-03-0005), HDAC 5 (M-003498-02-0005), HDAC 6 (M-003499-00-0005), HDAC 7 (M-009330-02-0005), HDAC 8 (M-003500-02-0005), HDAC 9 (M-005241-03-0005), HDAC 10 (M-004072-00-0005), p300 (M-003486-04-0005), CBP (M-003477-02-0005), PCAF (M-005055-00-0005) or control scramble siRNAs (D-001210-01-50). Transfections were performed in OptiMEM medium (Invitrogen) supplemented with 1% NEAA and 5% FBS. Knockdowns were assessed after 48 h using qRT-PCR and immunobloting analysis.

Discussion

Chapter VI – Discussion

This chapter aims to discuss the presented results for the (epi)genetic regulation of DEFB2 expression in the context of regulatory mechanism of inducible inflammatory gene expression. In more detail the role of the transcription factor NF-κB, the histone acetyltransferase p300 as well as the specific histone marks H3S10-P in association with MAPK pathways, H3K9-Ac, H3K27-Ac and H3K56-Ac will be discussed. Following the discussion there will be a short conclusion on hypothesis, followed by a brief perspective on the experimental level.

VI.1 Regulation of inducible inflammatory gene expression on the chromatin level

The inducible response to an inflammatory stimulus is complex and results in the orchestrated activation of many target genes with various functions, such as to alert the body to infection, fight invading pathogens, and repair damaged tissues. The outcomes of activation of those different genes can have opposing influence on the host physiology and homeostasis and therefore they have different regulatory requirements. Especially potentially harmful pro-inflammatory cytokines, which can have deleterious effects on the tissue when expressed at high levels over a long time period, have to be differentially regulated than antimicrobial peptides and tissue repair factors. Control of gene expression via the same signaling pathways, such as TLRs, NF-κB and MAPK, does not allow for differentiation in target gene expression and fine tuning of the defense response. Therefore another program of regulatory mechanism is needed to allow a single gene to be expressed or silenced according to its function, irrespective of other genes induced by the same signaling pathway. Evidence is increasing that epigenetic modifications mediate this level of regulation at individual promoters.

VI.1.1 Presetting the chromatin environment of NF-κB target genes

The transcription factor NF-κB is one of the key regulators of the inflammatory and immune response. The κB-binding site is estimated to be found $1.4x10^4$ times in the human genome (extrapolated from study by Martone and colleagues [695]) and these

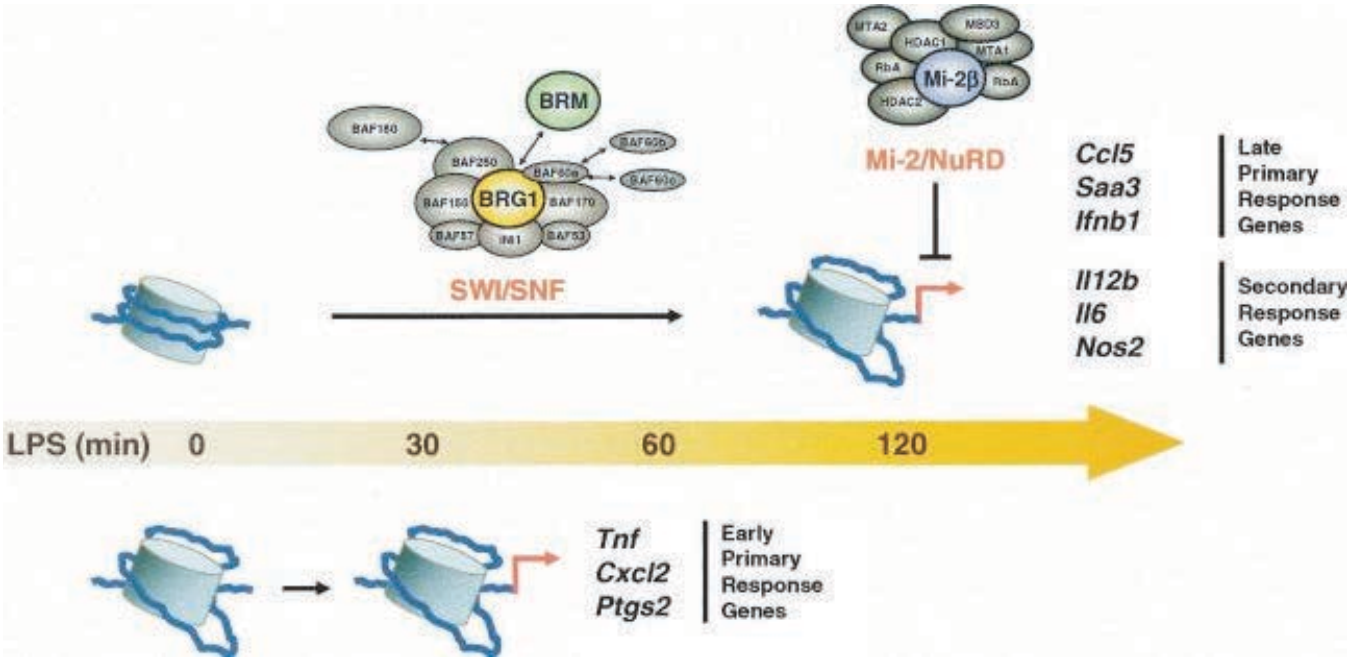


Figure 46: Requirements for chromatin remodeling on primary and secondary response genes. The figure depicts the selective requirement for SWI/SNF complexes at secondary response and late primary response genes, with Mi-2/NuRD negatively influencing these same sets of genes. Early primary response genes do not appear to be regulated by either SWI/SNF or Mi-2/NuRD complexes [581].

sites regulate two to three hundred NF-κB targeted genes. The number of p65 molecules entering the nucleus after a stimulus is estimated to be $1.5x10^5$ [696]. With this abundance it could be expected that NF-κB quickly finds its way to its binding sites and activated gene transcription. Yet this is not the case, and the kinetics of recruited p65 to target genes differs widely, between 10 minutes up to 2 hours [584]. This highlights the importance of additional pathways and coregulators to fine-tune NF-κB target gene expression. Nuclear presence is not enough and the targeted recruitment of p65 to a promoter integrates multiple inputs from simultaneously activated signaling pathways.

Two studies by Ramirez-Carrozzi and colleagues and Saccani and colleagues have shown that TLR-signaling induces recruitment of NF-κB and expression inflammatory genes in two waves, the primary response genes, which are further divided into early (eg. TNFα, MIP2) and late genes (eg. RANTES, IFNβ), and secondary response genes (eg. IL12b, IL6, Nos2) [581,584] (Figure 46). Moreover, several studies could show that there seems to be a presetting on the chromatin level for primary inducible genes. Ramirez-Carrozzi and colleagues could show that the expression of early primary response genes does not require chromatin remodeling by the SWI/SNF complex, in contrast to the expression of late primary response genes and secondary response genes [581] (Figure 46). The basal chromatin environment of primary induced genes is characterized by high levels of histone acetylation as well as occurrence of specific activating marks, such as H3S10-P, H3K9-Ac, H3K14-Ac and H3K4-me3, and association with promoter-proximal paused Pol II [579,607,697]. Overall the histone acetylation level is correlated with gene expression and several studies have described specific acetylation patterns at NF-κB target gene promoters in different cell lines upon inflammatory stimuli, with the main mediating HATs being p300, CBP and PCAF [698]. Interestingly, Saccani and colleagues found that different stimuli such as LPS vs TNFα, don't lead to hyperacetylation and activation of the same NF-κB target genes, highlighting the fact that there is differential chromatin modifying regulation probably mediated by different enzymes and cofactors.

Nothing has been described so far about the occurrence of these marks on the DEFB2 promoter and ChIP studies will give more insights into the chromatin state before and after stimulation. We can assume that the treatment with HDAC inhibitors

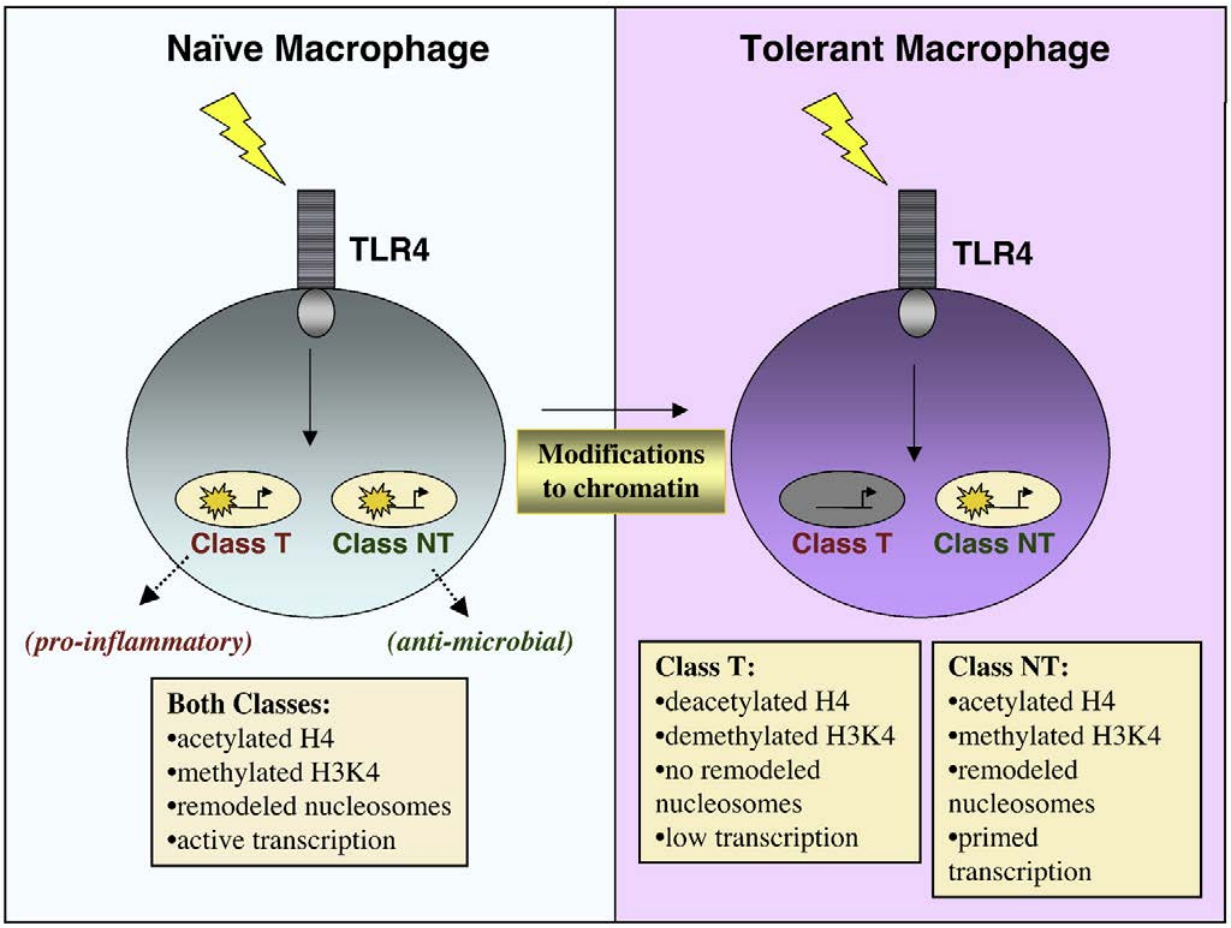


Figure 47: Induction of LPS tolerance in macrophages by chromatin modification. Following LPS stimulation of naive macrophages, both tolerizable (class T) and non-tolerizable (class NT) promoters recruit transcription factors and display increased histone acetylation, histone H3K4 trimethylation, and accessibility. In tolerant macrophages, class T promoters are deacetylated and inaccessible. Class NT promoters, on the other hand, are inducibly reacetylated, remethylated, and made accessible with faster kinetics upon a second stimulation with LPS [701].

transforms the DEFB2 promotor into an early accessible state, which enhances the NF-κB-dependent inducibility of its expression upon stimulation. Especially the kinetics and magnitude of transcription of pre-mRNA at the promoter is significantly enhanced within the first two hours, as shown by qPCR analysis. In fact Saccani and colleagues mention that these sort of promoters are able to recruit p65 without any stimulus, which could explain the increased basal expression in non-stimulated TSA treated cells [584]. Why this does not happen at the IL8 promotor is still unanswered. It can be envisioned that there is a differential protective mechanism, which inhibits such scenarios for IL8, as a boost of its expression beyond the normal level could have detrimental effects.

Interestingly, the occurrence of high levels of H3-Ac and H3K4-me3 correlated with high presence of CpG islands in those gene promoters [607], indicating a pre-disposing mechanism inscribed in the promoter sequence of primary response genes. Moreover, it was shown that CpG rich sequences have a lower affinity for nucleosome occupancy, thereby allowing for faster recruitment of the general transcription machinery and cofactors without the need for chromatin remodeling [607]. A simple analysis of the DEFB2 sequence shows that there is not a high abundance of CpG in the promoter region of this gene. The nucleosome density could be assessed by DNAse I hypersensitivity assays.

Moreover, the classification by chromatin environment is dependent on the cell type. IL6 was shown to be a remodeling dependent secondary response gene in murine macrophages, while its promoter was highly accessible in unstimulated murine embryonic fibroblasts and did not change upon stimulation. This suggests that the requirements for gene expression can be adjusted on the chromatin level according to the need in a specific cell type. Therefore it should be considered that the epigenetic regulation of the DEFB2 gene might be different in epithelial cells and immune cells, according to their different localization and roles in immune surveillance and defense.

Furthermore, also negative regulation of gene expression has been attributed to chromatin alterations. For example, the induction of LPS-tolerance in macrophages, and thereby the shut down of a subset of pro-inflammatory genes towards a second stimulation by LPS, was shown to be regulated via chromatin modifications. The major features of tolerized inflammatory genes was the loss of H4

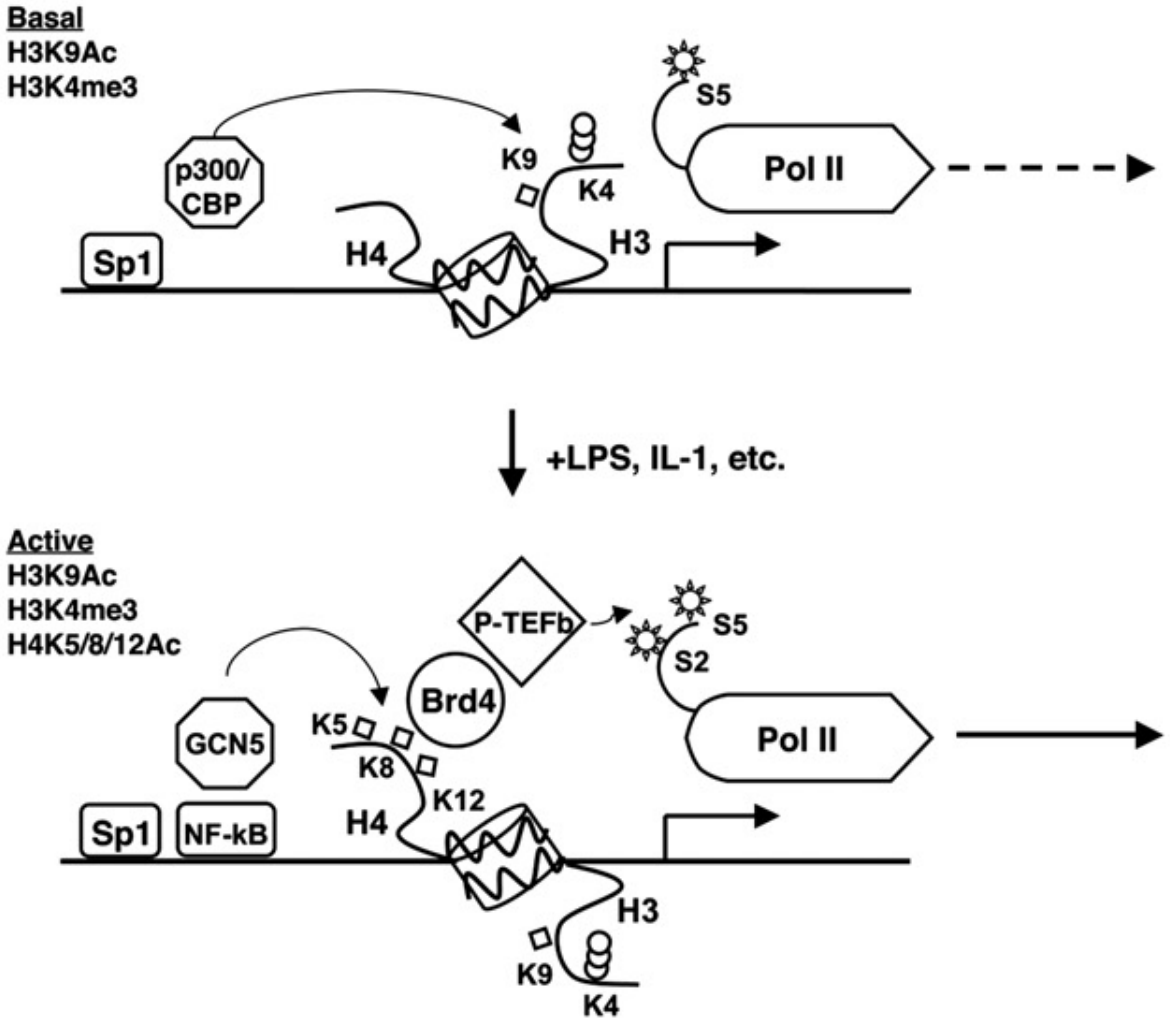


Figure 48: Model of LPS-induced primary response gene transcription. At the basal level Sp1 recruits Pol II and induced the generation of unspliced unstable transcripts. Upon LPS stimulation NF-κB and GCN5 are recruited, and acetylation of H4K5/K8/K12 is established. Those marks bind the cofactor Brd4, which in turn recruits P-TEFb to mediate the secondary phosphorylation of paused Pol II and thereby initiates productive transcription of target genes. Squares indicate acetylated residues, circles indicate methylated residues, and stars indicate phosphorylated residues [579].

acetylation and H3K4-me3, accompanied by a block of nucleosomal remodeling and loss of recruitment of nuclear p65 [699,700] (Figure 47). On the other hand, a group of non-tolerized antimicrobial genes maintained active chromatin structures and were still inducible upon a second stimulus, highlighting the separation of gene subsets simply be chromatin features [699]. Moreover, products of the first LPS-stimulation were shown to be responsible for the chromatin modifications, which on one hand shut down tolerized genes and on the other hand primed non-tolerized genes to even be induced with increased kinetics and magnitude. In addition, these data highlight the possibility to separate the expression of two sets of genes with different functions on the epigenetic level. The described boost in gene expression by chromatin alterations can be compared to our observation for the increase in kinetics and magnitude of DEFB2 gene expression upon TSA pre-treatment. We can speculate that the same kind of chromatin priming occurs in our case.

VI.1.2 The role of promoter-proximal paused Pol II

As mentioned above, many primary response genes are characterized by a basal level of paused Pol II at their promoter regions [579]. The constitutive transcription factor Sp1 was reported to be required for basal Pol II recruitment and induced the generation of unspliced unstable transcripts. This was shown to be necessary for the maintenance of the active chromatin state at primary response genes. Furthermore, Hargreaves and colleagues found that LPS stimulation of macrophages leads to the recruitment of HATs GCN5 and PCAF to primary response gene promoters. This correlates with the establishment of acetylation marks H4K5/K8/K12, which are in turn able to bind the cofactor Brd4 (Bromodomain-containing protein 4). Brd4 recruits P-TEFb to mediate the secondary phosphorylation of paused Pol II and thereby initiates productive transcription of target genes (Figure 48). This is possible to be related to the recruitment of NF-κB by Brd4, as suggested by Huang and colleagues [702]. And indeed inhibition of NF-κB abolished all the events downstream of initiation by LPS-treatment, emphasizing its role in the generation of functional full length transcripts [579]. The presence of promoter-proximal paused Pol II at the DEFB2 promoter in the basal state could be investigated by ChIP experiments. Furthermore, we could not observe a role for GNC5 and PCAF in the DEFB2 expression in our model, but the role of p300 in our setting might be redundant. The presence of

acetylation marks H4K5/K8/K12 and recruited Brd4 on the DEFB2 promoter could also be investigated by ChIP experiments.

Studies in *Drosophila* also described the role of the histone mark H3S10-P in the release of promoter-proximal paused Pol II. This chromatin modification is a hallmark of active genes in *Drosophila*, especially at the highly active heat shock loci upon temperature increase [703]. The MSK1/2 homologous kinase JIL1 was found to phosphorylate H3S10 on target genes after transcription initiation and thereby facilitate the release of promoter-proximal paused Pol II and productive elongation [704]. JIL1 was shown to be required for the expression of the majority of *Drosophila* genes. In JIL1 mutants transcription factors and Pol II are still recruited, but P-TEFb is lacking, indicating a missing second phosphorylation of Pol II mediated by this complex. It is possible that H3S10-P plays a similar role in mammalian cells.

VI.1.3 Repression of inducible genes by HDAC complexes

The question remains what keeps primary response genes inactive in the unstimulated state? Most Interestingly, many of those genes were found to be associated with repressor complexes containing HDACs, such as NCoR/HDAC3 and CoREST/HDAC1. It was speculated that those enzymes keep H4K5/K8/K12 in a deacetylate state, thereby preventing the recruitment of Brd4 and the subsequent release of Pol II into elongation. Moreover, NCoR was shown to be specifically recruited to NF-κB and AP1 regulated targets by p50/p50 and c-Jun/corepressor dimers [705,706]. Hargreaves and colleagues found that p50 was most abundant in primary response promoters in the absence of p65 [579]. Since DEFB2 is regulated by NF-κB and AP1 it is imaginable that the expression of DEFB2 is normally hindered by repressive complexes associated with HDAC enzymes, whose function is negated due to the treatment with HDAC inhibitor.

VI.2 Regulation of inducible inflammatory gene expression via NF-κB

As already mentioned, NF-κB is on of the main transcription factors regulating the stimulus induced expression of inflammatory and immune genes. Its activity can be regulated on multiple levels: First by activation in the cytoplasm, then by post-translational modifications, by interaction with cofactors, and nonetheless, by the possibility of the formation of different dimers. All these possibilities will be discussed after a brief introduction into the important role of this factor, not only in inflammation and defense, but also in homeostasis of the gut.

VI.2.1 The role of NF-κB in intestinal homeostasis

The transcription factor NF-κB is one of the major regulators of inflammatory gene expression. Therefore, dysregulation of its activity can lead to a range of inflammatory pathologies. The role of NF-κB in physiology and disease has been widely studied in genetic mouse models possessing cell-specific increased or inhibited NF-κB activity (as complete KO mice suffer from severe inflammation and early post-natal death) [707]. Unexpected observations have been made in mice carrying alterations in NF-κB expression in IECs. One would expect that overexpressed NF-κB would cause severe inflammation, but only mild mucosal inflammation was observed upon sustained increased NF-κB activity in IECs. Besides, these mice were strongly sensitized to intestinal challenges such as chemical induction of colitis with dextran sulfate sodium, inflammation triggered by LPS or TNF, and infection with enteric pathogens [708,709]. On the other hand, inhibition of NF-κB activity should have an anti-inflammatory effect, as widely observed for immune cells. On the contrary, IEC-specific knockout of members of the IκB kinase complex and thereby blocking of NF-κB activation lead to spontaneous intestinal inflammation [710]. Knockout of either IKKα or IKKβ did not lead to development of inflammation and only partially inhibited NF-κB activation, probably due to functional redundancy. But knockout of a combination of IKKα and IKKβ, or of the NF-κB essential modulator (NEMO) lead to development of severe chronic colitis, due to death of IECs, disruption of the epithelial barrier and bacterial invasion of the mucosa. Interestingly, IEC-NEMO knockout mice showed decreased expression of defensins, which has also been related to Crohn's disease in humans [314]. Moreover,

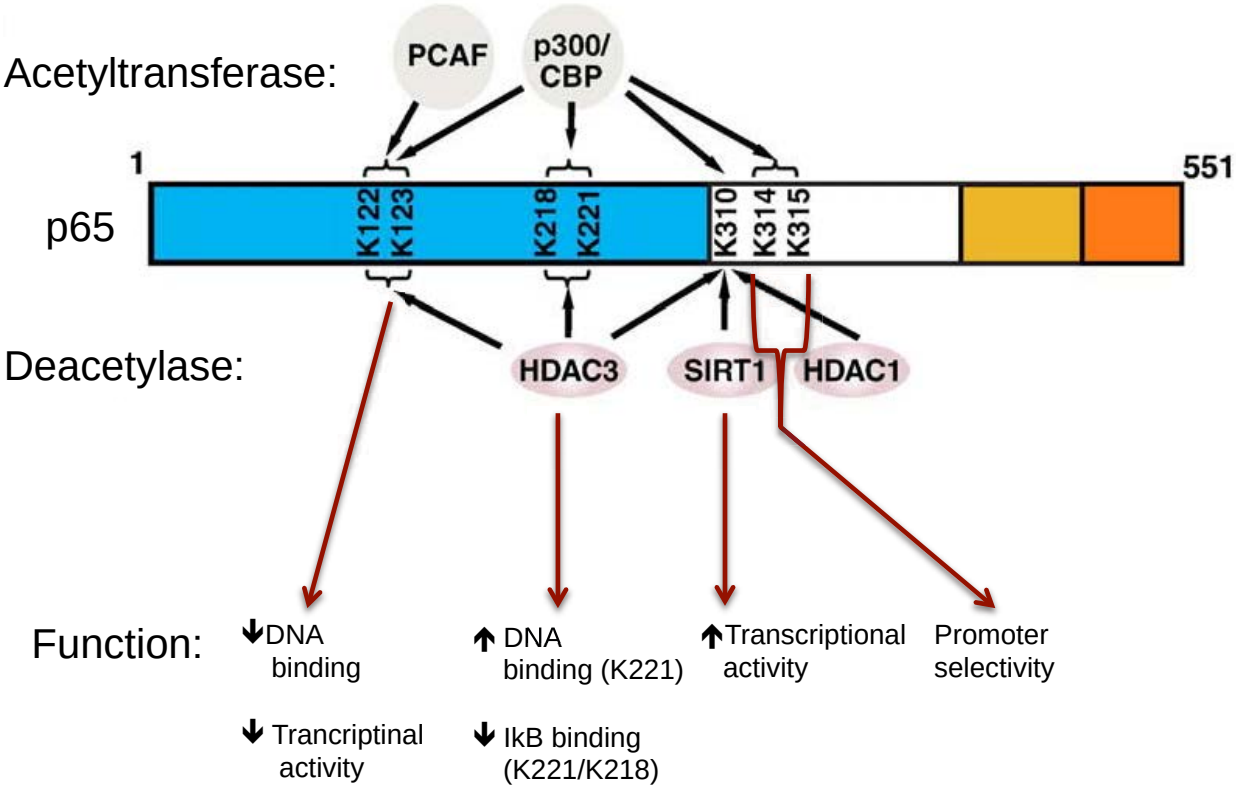


Figure 49: Acetylation of p65 and its different outcomes on NF-κB activity. The p65 subunit of transcription factor NF-κB is reversible acetylated on several lysine residues (K) by HATs PCAF and p300/CBP. Acetylation of these lysines can have various influences on the activity of NF-κB as indicated. Acetylations are removed by HDAC1 or 3 and SIRT1. (Adapted from [662])

humans carrying a mutation in the NEMO gene show colitis as one of the symptoms [711]. IEC-specific knockout of the NF-κB subunit p65 lead to spontaneous intestinal pathology and early death in only 10% of mice and did not affect intestinal health of adult mice [712]. This implicates that other subunits can partly compensate for the loss of p65.

These findings highlight the role of NF-κB as a major messenger between microbial communication via PRRs and the epithelial response, which regulates barrier integrity and homeostasis [713]. The question of how NF-κB can play this dual role as trigger of inflammation and maintenance of tissue homeostasis is highly interesting. Many studies focused on the ability of NF-κB to differentially regulate target gene expression, with the help of alterations on the chromatin level. This will be discussed in greater depth in the following chapters.

VI.2.2 Acetylation of NF-κB regulate its function and activity

On a first level, the stimulus-induced activation of cytoplasmic NF-κB is tightly regulated by its inhibitor IκB (Figure 35). Once activated and translocated to the nucleus, a second level of regulation kicks in: the reversible addition of extensive post-translational modifications. Especially the subunit p65 can be phosphorylated, acetylated, methylated and ubiquitinated on various residues. These modifications are in cross-talk with each other, mediate the binding of cofactors or corepressor, regulate the strength of DNA binding and the duration of NF-κB nuclear activity as well as target gene specific transcription [662]. This post-translational “NF-κB code” resembles not only the scheme used by histone modifications, but also shares some of its key enzymes, such as HATs and HDACs.

As NF-κB is one of the key transcription factors responsible for DEFB2 expression, and acetylation seems to play a major role in the regulation of this gene, we were highly interested in the regulation of p65 by acetylation. So far 7 lysine residues acetylated by p300/CBP and PCAF have been identified within the p65 subunit: lysines 122, 123, 218, 221, 310, 314 and 315 (Figure 49). Multiple studies have aimed to allocate distinct functions to these lysine-acetylation marks. Chen and colleagues found that acetylation of K221 enhances the DNA binding of NF-κB [714]. Together, acetylation at K221 and K218 prolong NF-κB nuclear activity by blocking its association with the inhibitor IκB, which is one of the NF-κB target genes, and is

rapidly produced in a negative-feedback loop. On the contrary, acetylation of K122 and K123 were shown to negatively regulates NF-κB-mediated transcription by reducing p65 binding to κB elements on DNA, and thereby facilitate its removal by IκB and removal from the nucleus [715].

A special role in transcriptional activity is allocated to acetylation of K310. Acetylation of this residue was shown to be required for full transcriptional activity of NF-κB and functions on two regulatory levels. First, Huang and colleagues could show that p300-mediated acetylation of K310 subsequently recruits the coactivator Brd4, which in turn mediates phosphorylation of Pol II via P-TEFb and stimulates transcription of NF-κB target genes [702]. Furthermore, they could show a differential recruitment of p300 as well as differential requirement for P-TEFb recruitment to target gene promoters after TNF stimulation of human lung carcinoma cells. How this differential recruitment is regulated remains undescribed, but a scenario involving acetylated histone marks as described by Hargreaves and colleagues can be envisioned [579]. Secondly, acetylation of K310 inhibits methylation of K314/315 by the methyltransferase Set9, which would lead to ubiquitination and degradation of chromatin associated p65 [716]. Several studies could show that TSA treatment prolonged nuclear presence of p65 and enhanced its DNA binding activity, possibly by enhances acetylation of this factor [717-719]. We could show by Western blot that overnight pretreatment with TSA strongly enhances the acetylation of p65 on K310 in IECs. Acetylation of other residues is expected, but could not be shown due to the lack of availability of commercial antibodies. It can be hypothesized that the increase acetylation of p65 enhanced its presence and activity in the nucleus and this could partly explain the strong increase of DEFB2 expression upon HDACi treatment. If this is the case in our model will have to be investigated by immunofluorescent staining for p65 or Western blot of nuclear extracts. ChIP analysis of the recruitment of p65 and p65K310-Ac to the DEFB2 and IL8 promoters will give more insight into the role of NF-κB in the differential expression of those genes upon HDAC inhibitor treatment.

At this point we can only speculate about the differential influence of p65 acetylation on DEFB2 and IL8 gene expression. In this regard, two studies described the role of acetylation of p65 K314 and K315 in the differential regulation of expression of specific sets of NF-κB target genes in response to TNFα stimulation [720,721]. They identified a few genes, which showed either increased or decreased

expression upon site-specific mutations creating acetylation mutants and subsequent whole genome microarray analysis after TNFα stimulation in mouse embryonic fibroblasts. AMPs were not described in this data set. The acetylation of lysines, which convey promotor specific targeting, such as K314 or K315 can be envisioned, but could not be investigated in the presented work due to lack of available antibodies.

The p50 subunit can be acetylated on lysines 431, 440 and 441 by p300 [722,723]. Biological relevance for p50 acetylation is less clear. Acetylation is increased after LPS or TNFα stimulation by p300 and has been associated with increased promotor binding and transcriptional activity. We did not investigate p50 acetylation in the presented work.

VI.2.3 Repression of NF-κB target genes by HDACs

Additionally, to the role of acetylation by HATs, also the role of deacetylation by HDACs in the regulation of NF-κB activity has to be considered. HDAC3 was shown to be responsible for the removal of acetylations on all known lysines of p65 and is a switch for IκB-dependent termination of NF-κB signaling [717] (Figure 49). HDAC1 and SIRT1 have been additionally reported to deacetylate K310 and decrease p65 transcriptional activity [724,725]. Moreover, Ashburner and colleagues found that the low level of nuclear p65 in unstimulated cells is associated with the corepressor complex HDAC1/HDAC2, via binding of HDAC1 with the Rel homology domain, and thereby unable to bind to DNA target sequences [726] (Figure 50). Furthermore, they showed that TSA treatment results in an increase in both basal and induced expression of an integrated NF-κB-dependent reporter gene, as well as the NF-κB target gene IL8, in mouse embryonic fibroblasts and HeLa cells. This indicates that nuclear HDAC-bound p65 is released and transcriptionally active upon HDACi treatment. This observation could also account for the increased DEFB2 expression upon HDACi treatment in the basal state.

But not only nuclear p65 sequestration by HDACs is keeping target gene expression in unstimulated cells in check, there is also the report of binding of nuclear p50 dimers which recruit HDAC1 to the promoter regions of a subset of NF-κB target genes [628,727]. The p50 subunit is predominant in the nucleus, as it cannot be bound and exported by the inhibitor IκB.

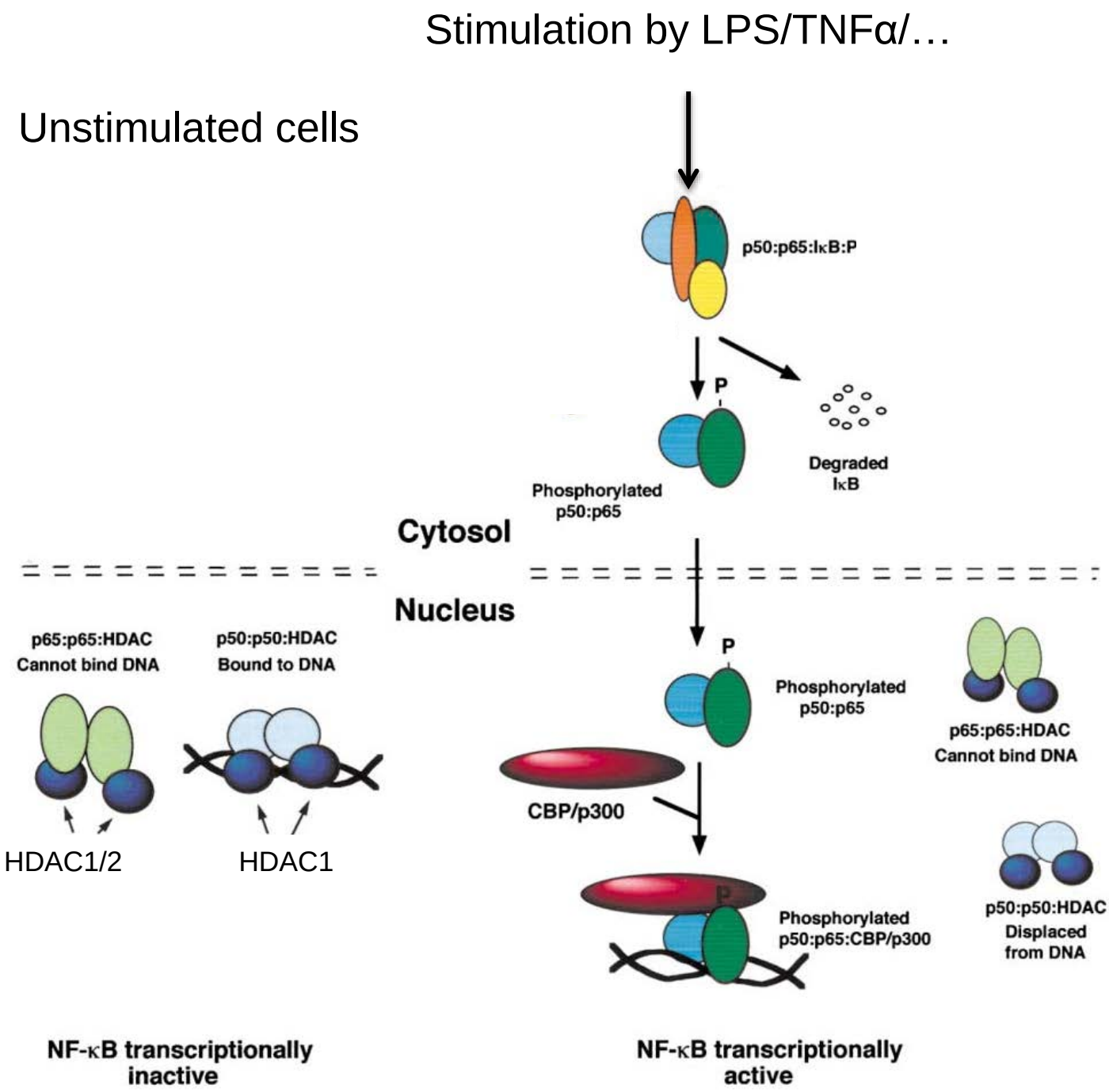


Figure 50: Repression of p65 and NF-κB target genes by HDAC complexes. In unstimulated cells (left), p50 or p65 homodimers are bound to HDAC1. While only p50/HDAC1 can bind to DNA at NF-κB target genes, both complexes are transitionally inactive. Inflammatory stimuli induce phosphorylation of cytoplasmic p65/p50 heterodimers and their release form IκB. Those activated dimers translocate into the nucleus and are able to associate with coactivators CDB/p300 and to displace p50-HDAC-1 complexes from DNA. This mechanism insures that only signal-induced, phosphorylated NF-κB drives target gene expression. (Adapted from [727])

Nuclear p50 homodimers were also found associated with HDAC1 and on the contrary to p65/HDAC1 they can bind to specific NF-κB target genes and repress their expression in unstimulated cells. Upon a stimulus cytosolic p65/p50 shuttles to the nucleus and is able to replace the repressive p50/HDAC1 complex to activate gene expression as its affinity for the consensus-binding site is much higher [728]. This replacement was shown for example for the INFβ promotor upon virus infection [729]. The importance for p50/HDAC1 mediated repression was emphasized by treatment of murine blood peripheral lymphocytes with the HDACi TSA (20-100 nM), which induced the expression of IL6 and iNOS in unstimulated cells [727]. Additionally, TSA treatment increased the acetylation of H4 on the IL6 promoter. ChIP experiments showed binding of p50/HDAC1 to the IL6 promoter in unstimulated cells. Furthermore, the inducing effect of TSA on IL6 and iNOS expression was lost in p50-/- mice. It can be envisioned that p50/HDAC1 associates and repressed the DEFB2 promoter in unstimulated cells and that this repression is revoked upon TSA treatment. This could explain the increase of basal expression of DEFB2 in non-infected cells. If the p50 subunit plays a role in this expression could be investigated by targeted knockdown of this protein. The presence of p50/HDAC1 complex and p50 alone at the DEFB2 and IL8 promoter will have to be evaluated by ChIP.

VI.2.4 Phosphorylation of p65 and cofactor binding

Not only acetylation directs the activity of NF-κB, but also phosphorylation plays an important role for the interaction with coactivators. Stimulus-induced phosphorylation of p65 at S276 and S536 by PKA or MAPK was shown to be necessary for efficient recruitment of the coactivators CBP/p300 and enhances acetylation of K310 [730,731] (Figure 49). The ability to bind coactivators is lost in a knock-in mouse mutant, which carries a non-phosphorylatable version of p65 [732]. ChIP assays revealed normal recruitment of this p65 mutant to promoters of target genes such as IL6 and IκBα, but reduced recruitment of HATs and therefore less acetylation of H3. This went hand in hand with the observation of increase recruitment of HDAC3 to those promoters. Interestingly, the expression of target genes was differentially influenced: Certain genes were completely blocked (e.g. MIP2), while others were relatively unaffected (e.g. IκBα and Cox2) or partially affected (e.g. TNFα, MCP1 and IL6). This study shows the differential requirement for cofactors such as p300 at NF-κB target genes, and thereby suggests that different cofactors are specific for the expression of certain

target genes. This goes hand in hand with our observation that DEFB2 shows a stronger requirement for p300 as compared to IL8, indicated by the 10-fold stronger reduction in expression upon chemical inhibition of p300.

VI.2.5 The role of NF-κB dimers in differential gene expression

The NF-κB family consists of five members, which can form multiple hetero and homo dimers. Studies performed in knockout mice and cells lacking individual NF-κB proteins showed that each NF-κB family member has a specific role in the induction of target genes [617,620]. Some dimers are uniquely required for the expression of specific target genes. The expression of IL12p40 in LPS stimulated murine macrophages for example specifically requires c-Rel homodimer binding [733] and p52:RelB dimers are necessary at the promoters of CXCL13 and CCL19 [734]. The involvement of p65/p50 at the DEFB2 promoter has already been describe, whereas nothing is known about the binding of other dimers. siRNA knockdown of p65 did not completely abolish the expression of DEFB2 in IECs. This could on one hand be due to incomplete knockdown and transcriptional activity of the p65 residual pool or it could be that other subunits play a role in the scenario of DEFB2 expression. The chemical inhibitor BMS-345541 blocks the activation of IKK and therefore, as well the canonical as the non-canonical NF-κB pathway [735]. The complete inhibition of DEFB2 expression upon inhibitor treatment shows therefore that NF-κB is essential, but does not allow differentiation between subunits. The investigation of recruitment of different dimers upon TSA treatment and infection on the DEFB2 and IL8 promoter by ChIP could give important insights into the role of different NF-κB dimers in this setting. It is possible that different dimers mediate the differentiation between DEFB2 and IL8 upon HDACi treatment. The question what conveys dimer specific regulation of distinct target genes is not easy to be solved and certain possibilities will be discussed here.

It can be imagined that preferable binding to specific derivates of the canonical κB site play a role, as well as affinity to shared binding sequences. A recent study by Siggers and colleagues re-evaluated DNA-binding site recognized by different NF-κB dimers in a protein-binding microarray containing more than 3000 potential κB binding sites [736]. Overall, they were able to identify a differential preference for kB site lengths for c-Rel and RelA homodimers (9 bp), heterodimers (10 bp), and p50 and p52 homodimers (11 bp). Moreover, binding was directed by a single consensus

half site. For homodimers, they found that c-Rel recognizes all κB-binding sites with a much higher affinity than p65 homodimers, despite highly correlated binding profiles. Therefore, to outcompete c-Rel, p65 homodimers need to rely on unique alternative mechanisms, such as coactivator interactions. p50 and p52 homodimers were found to recognize very similar sequences and show differential sequence preference as compared to other dimers.

Surprising were the results for heterodimers, where they found highly overlapping binding specificity of all NF-κB heterodimers and considerable potential for competitive binding. These results indicate that selective function of each heterodimer may not be achieved via dimer-specific recognition of κB motifs but likely by alternative mechanisms, such as dimer-specific interactions with co-regulatory proteins and other transcription factors. Nevertheless, it cannot be excluded that relatively small differences in DNA-binding specificities can have important functional consequences.

In addition to the overlapping recognition of binding sites, relatively small affinity differences between heterodimers were observed. It is not known if an affinity threshold to activate gene expression exists, but high-affinity binding sites for NF-κB and other factors were shown to lead to stronger transcription than low-affinity sites [737]. The role of affinity is Additionally, questioned by a study from Bosisio and colleagues, who showed that while *in vitro* NF-κB dimers have a very high affinity for κB sites and generate very stable complexes with a half-life of 45 minutes, the *in vivo* situation is completely different. They found that NF-κB is only transiently immobilized and the complete turnover of NF-κB on activated chromatin occurs in less than 30 seconds [738]. The fast dynamics of NF-κB binding and unbinding generates a dynamic equilibrium between promoter-bound and nucleoplasmic NF-κB dimers and oscillations in promoter occupancy and transcriptional activity.

Others also implied the role of transcription factor kinetics and different activation dynamics in target gene specificity. For example, c-Rel and p65 complexes have been reported to be differentially exported from the nucleus and may be subject to differential degradation as the NF-κB response is attenuated [739,740].

One possibility for dimer specific recruitment of cofactors is the observation that different dimers adapt different conformations when bound to target gene

sequences. p50 homodimers for example were found to change confirmation upon binding to certain target gene motifs and only then to induce their transcription [741]. A later study by Leung and colleagues strengthens the theory that the consensus-binding site may only weakly determine the ability of a particular dimer to bind. Instead, it rather affects the configuration of the bound dimer and thereby determines which coactivators will form productive interactions [742]. Furthermore, it was shown that the same promoter needed coactivators for the induction in response to one stimulus, but not to another, such as the need of IκBξ at the IL6 promoter stimulated by LPS versus TNFα [743]. Additionally, to the investigation of actual recruitment of different subunits, it could be interesting to compare the sequences of the κB binding sites at the DEFB2 and IL8 promoter. Differences in sequence might explain differential requirement for cofactors, such as for example p300.

VI.3 The possible role of enhancers in DEFB2 expression

Promoters are located at the 5' ends of genes, surrounding the transcriptional start site (TSS), and mark the initiation point of transcription. Enhancers on the other hand contribute to the activation of their target genes from positions upstream, downstream or within a target or neighboring gene [744]. Some enhancers are even located thousands of kilo bases away from their target or even on a different chromosome, yet they are able to regulate timing and levels of the expression of their targets.

Distal control regions have been shown to play a role in the expression of inducible immune genes, such as IL2 and IL4 in T cells [745,746]. Furthermore, the LPS-dependent expression of IL12p40 in murine macrophages by the c-Rel homodimer requires induced nucleosome remodeling at the promoter and distal enhancer region at the same time [747,748]. It is possible that DEFB2 is partly regulated by such enhancer elements, which are activated upon acetylation after HDACi treatment.

The identification of enhancers throughout the genome has been incredibly aided by the discovery of specific enhancer-associated chromatin signatures, such as the histone mark H3K4me1 combined with low amounts of H3K4me3 [749]. Additionally, constitutive as well as inducible localization of p300 to those domains was shown in untreated and IFNγ stimulated HeLa cells [749], and LPS stimulated

murine macrophages [750]. A consecutive study Additionally, identified frequent acetylation of H3K27 at active enhancers [561]. Furthermore, studies with other HDACi have shown that they can influence the level of H3K27 acetylation. ChIP analysis of butyrate treated T cells showed a marked specific increase in H3K27-Ac at the Foxp3 promoter and its intronic enhancer CNS1 (conserved non-coding sequence 1) [751]. The fact that we observe the involvement of p300 in DEFB2 expression, as well as the induced increase in H3K27-Ac upon HDACi treatment points towards the possibility of enhancer involvement. Nothing has been described so far in this regard for the DEFB2 gene.

One example for the regulation of gene expression by a proximal promoter and a distal enhancer in NF-κB target genes has been described for the monocyte chemo attractant protein 1 gene (MCP1) in response to TNFα. In unstimulated cells, only the distal enhancer region is accessible to transcription factors. Upon stimulation with TNFα, NF-κB binds to the distal enhancer region and recruits CBP and p300 [752]. Those HATs modify and open the local chromatin structure until it can form a stable interaction with the proximal promoter element. In this scenario, an increase in histone acetylation at both the distal and proximal regions as well as within the intervening sequences have been described [753]. This interaction finally enables binding of Sp1 and the general transcription machinery at the promoter as well as enhances the binding of the expression coactivator CARM1 to the distal enhancer region. This study shows a model in which two independent chromatin states exist at the promoter and the distal enhancer, thereby allowing for prevention of inappropriate gene activation at the promoter and rapid induction through the distal enhancer at the same time.

In *Drosophila*, the affinity of binding sites for the NF-κB protein Dorsal correlates with the activity of gene transcription. Enhancers bearing low affinity binding sites, require higher amounts of Dorsal for their activation, which sets a kind of threshold for gene transcription [754]. It could be envisioned that a DEFB2 enhancer, which contains a low affinity p65-binding site, is activated by the increased nuclear presence of acetylated p65 upon HDACi treatment.

VI.4 The role of specific histone marks in HDACi enhanced DEFB2 expression

As we observe a strong increase in the acetylation of H3K9, H3K27 and H3K56 upon TSA treatment, it is intriguing to speculate about their role in the enhanced DEFB2 expression. Moreover, it has been described that p300 is able to acetylate all these marks [755,756]. Bedford and colleagues even showed that knockout of CBP/p300 in mouse embryonic fibroblasts leads to the loss of all H3K27-Ac [757]. As already mentioned, H3K27-Ac, H3K9-Ac and p300 are prominent marks of enhancers [561,757]. Additionally, H3K27-Ac and H3K9–Ac are commonly found surrounding the TSS on promoters of active genes [555]. A study by Agalioti and colleagues showed that H3K9-Ac together with H3K14-Ac is critical for the recruitment of the general transcription factor TFIID at the IFNβ promoter upon virus infection of HeLa cells and therefore the initiation of transcription [758]. Furthermore, acetylation of those marks prevents their competitive methylation, which would turn them into the repressive marks H3K27-me3 and H3K9-me3 [759]. All these findings support the possibility of an activating role of these marks at the DEFB2 promoter and possible enhancer. If they occur there specifically enhanced after TSA treatment will have to be established in ChIP experiments.

On the contrary, not so much is known about the function of K56 acetylation, which lies not on the tail, but the globular core domain of histone H3. Recent work in yeast showed that H3K56-Ac has a critical role in packaging DNA into chromatin following DNA replication and is involved in DNA damage repair [760,761]. Another study showed that H3K65-Ac is involved in promoter chromatin disassembly in yeast, which is also a widely used mechanism to regulate transcriptional induction in eukaryotic cells. They suggest that there is an equilibrium of H3/H4 disassembly and reassembly at promoters that is regulated by the level of H3K56 acetylation to facilitate rapid changes in gene expression [762]. Not much has been described about the role of this histone mark in eukaryotic cells so far. Das and colleagues could show that H3K56-Ac is also involved in DNA repair in *Drosophila* and human HeLa cells. The level of this marks increase at repair sites in a concentration dependent manner with the treatment of DNA damaging agents [756]. Moreover, they could show that CBP and p300 are responsible for the acetylation of H3K56 in human cells. Work

coming from Schneider and colleagues Furthermore, indicates that this mark is associated with actively transcribed genes and could be involved with the elongating Pol II in yeast and *Drosophila* [763]. One study in human adipocytes set out to locate H3K56-Ac over the complete genome. They also found this mark primarily located around TSS and Additionally, reported that it was associated with a generally higher expression of those genes [764]. Interestingly, they found a 87% overlap of H3K56-Ac with the results for H3K27Ac coming from another study in adipocytes [765]. It can be imagined that the acetylation of this core lysine is also involved in transcriptional initiation in eukaryotic cells and could support the expression of DEFB2 in our model.

VI.5 Regulation of inducible inflammatory gene expression via MAPK pathways

MAP kinases are important transmitters of inflammatory stimuli and are activated concurrently with NF-κB. Moreover, they were found in promoter-bound transcription complexes, together with Pol II and general transcription factors, and can activate those via phosphorylation [633]. They not only mediate recruitment of remodeling complexes and histone modifying enzymes, but are even capable of directly modifying chromatin themselves [766]. All these features make them an interesting player in the regulation of inducible gene expression [767].

VI.5.1 The role of H3S10 phosphorylation in inducible gene expression

One of the outcomes of stimulus induced MAPK signaling is the direct downstream phosphorylation of histone 3 at the serine 10 residue (H3S10-P) (Figure 50). Different stress stimuli were shown to induce this mark at selective promoters of primary response genes and thereby regulate inducible gene expression in mouse fibroblasts. The signaling cascade was shown to go via ERK or p38 followed by MSK1/MSK2 activation, which directly phosphorylate S10 [766,768]. Inflammatory stimuli, such as LPS, as well activate p38 and downstream kinases to phosphorylate H3S10 in human primary DCs. This in turn enhances the accessibility of NF-κB binding sites in a selected subset of target genes (for example IL6, IL8, IL12p40, MCP1) [697]. But not all genes were found to be activated in the same manner. Moreover, there was also p38-independent phosphorylation of H3S10 as well as

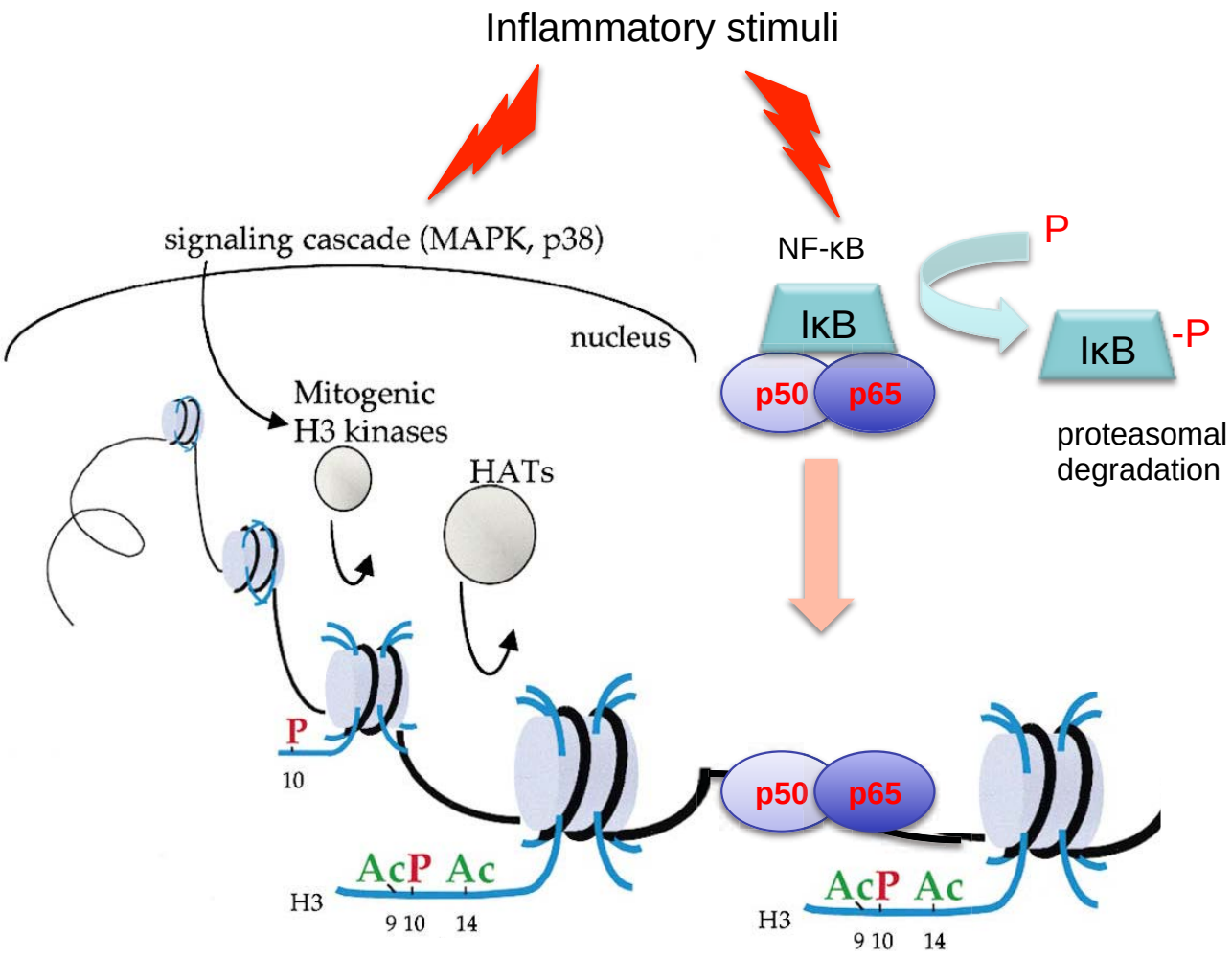


Figure 51: MAPK-signaling induced H3 phosphorylation on serine 10 and following acetylation events. Activation of MAP kinase pathways results in the activation of mitogenic H3 kinases (eg. MSK1/MSK2) and leads to rapid and transient phosphorylation of H3 at serine 10. H3S10-P promotes the recruitment of HATs and subsequent acetylation of nearby residues H3K9 and H3K14. Together, these events can facilitate transcription of the immediate-early genes by concurrent NF-κB activation. (Adapted from [770])

gene activation without prior H3S10 phosphorylation, indicating a differential regulation and requirement of this histone mark in the activation of NF-κB target genes [697].

Additionally, other groups could show that H3S10-P can influence the surrounding histone residues and thereby direct specific outcomes. For example H3S10-P was found to increase the binding and activity of several HATs, including p300, and thereby promotes subsequent acetylation of H3K14 and H3K9, which also correlates with transcriptional activation of targeted promoters [769] [771] (Figure 51). Interestingly, this depends on the target gene, as not all HATs have increased activity towards H3S10-P, and the same HAT may require H3 phosphorylation for maximal activity on some promoters but not on others [769].

As already mentioned, we observe a strong increase in general H3S10-P upon TSA pre-treatment in our model. If TSA activates MAPK in our model and if so, which one, needs to be established by Western Blot. Furthermore, the involvement of different MAPK could be investigated by the use of specific inhibitors. Other studies showed that also IKKα can mediate phosphorylation of H3S10 at NF-κB-responsive promoters upon cytokine stimulation [772,773]. As we see a decrease in the protein level of the NF-κB inhibitor IκB by Western blot upon TSA treatment in non-infected cells this could indicate an activation of the IKK complex, which might target H3S10. If this mark occurs specific at the DEFB2 promoter and correlates with the presence of a certain MAPK or IKK could be investigated by ChIP analysis.

Furthermore, it can be envisioned that H3S10-P recruits p300 to the DEFB2 promoter. Also this possibility will have to be established by ChIP experiments. Additionally, recruited p300 could further facilitate remodeling of chromatin at the DEFB2 promoter and allow for enhanced binding of NF-κB. The possible discrepancy between DEFB2 and IL8 could be mediated via differential recruitment of kinases, as it was shown in yeast where the MAPK Hog1 is recruited by sequence specific stress related transcription factors to individual target genes [774].

The importance of H3S10-P dependent regulation of gene expression in the immune response is underlined by the fact that pathogens have evolved mechanisms to inhibit H3S10-P and thereby gain advantage over the host's protective force. This is the case for example for *Shigella flexneri*, which uses the secreted effector OspF

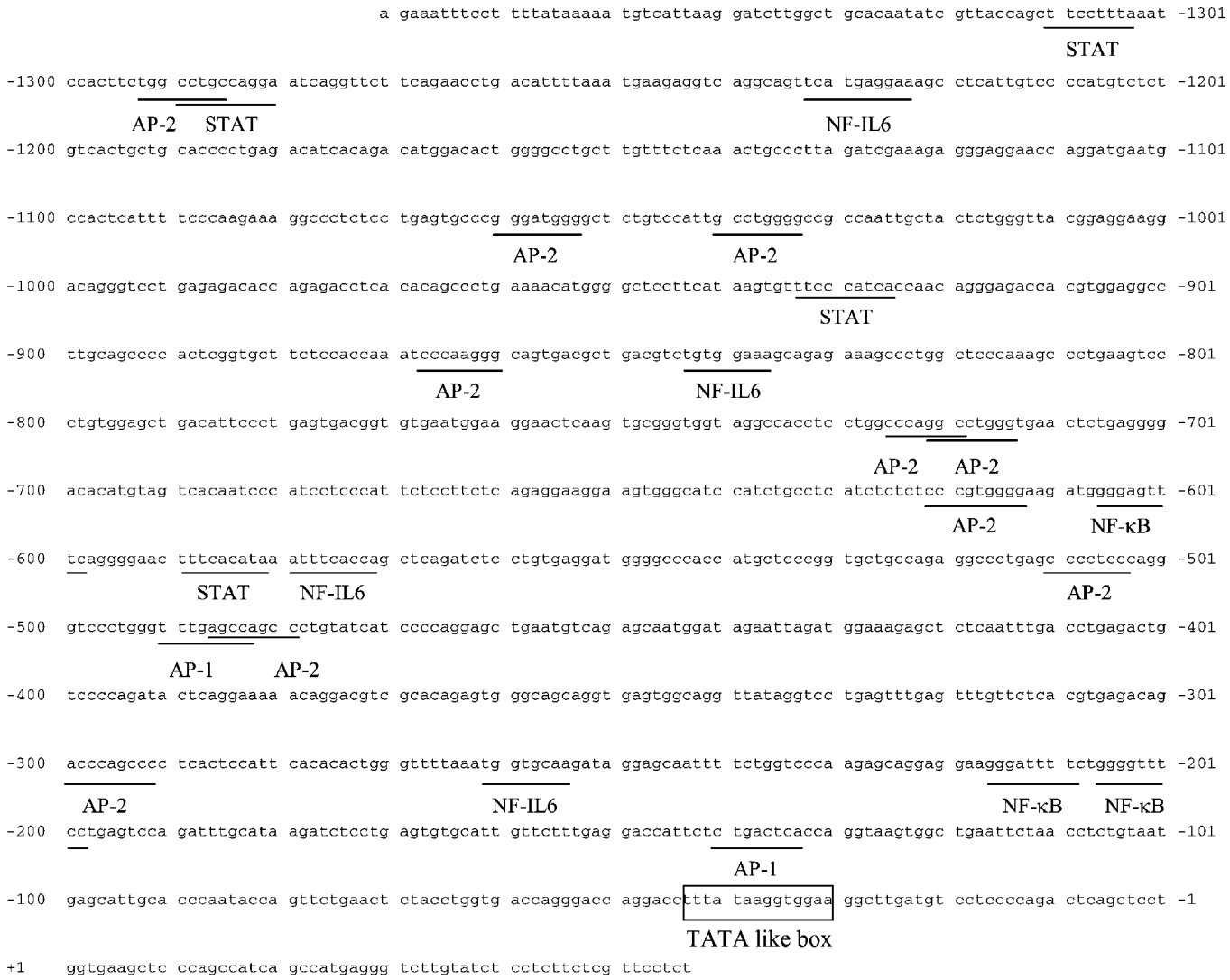


Figure 52: Putative transcription factor binding sites of the DEFB2 gene. This scheme shows the nucleotide sequence of the 5'-flanking region of the DEB2 gene and putative transcription factor binding sites (underlined), and the TATA-like box as determined by computational analysis [629].

to dephosphorylate p38 and ERK and thereby block the downstream phosphorylation of S10 at promoter of a subset of genes involved in immune response [775]. Especially IL8 expression was impaired in this scenario. Also *Listeria* LLO was shown to decrease H3S10-P, the involved signaling cascade has not been identified [776].

VI.5.2 The role of AP1 in AMP expression

The transcription factor AP1 has been implicated in the expression of LL37 and DEFB2. Interestingly, some studies were able to link HDAC inhibitors to the induction of AP1. A study by Nepelska and colleagues investigated the effect of bacterial culture supernatants on signaling pathways in IECs. They found that the amount of butyrate in the supernatant correlated with the induction of the AP1 pathway and that butyrate activates AP1 via ERK1/2 [777]. Furthermore, they found that TSA activates AP1 in a luciferase reporter cell line of IECs. In a step further, a study in lung epithelial cells showed that butyrate increases cathelicidin expression in an AP1 dependent manner, as indicated by mutation of the AP1 binding site in the cathelicidin promoter [537]. Blockage of all three MAPK pathways inhibited the effect of butyrate on cathelicidin expression. On the contrary, a study by Schauber and colleagues describe that butyrate and TSA treatment induce the expression of LL37 in IECs and that this induction was completely blocked by inhibition of the MEK/ERK pathway, but independent on AP1 [534]. The link between HDAC inhibitors and MAPK/AP1 activation seems to be not clear yet. Since DEFB2 possesses an AP1 binding site, there is a possibility that this transcription factor plays a role in the TSA-induced overexpression of DEFB2 (Figure 51).

VI.6 The combination of transcription factors in DEFB2 expression

We could show by knockdown and chemical inhibition that p65 is necessary for the expression of DEFB2 in IECs upon *E. coli* stimulation. This is in concordance with data coming from other groups [627]. Still we cannot exclude that other transcription factors play a role in DEFB2 expression, as there are multiple putative binding sites located in the 5'-flanking region (Figure 52). A study by van Essen and colleagues showed that binding of p65 is necessary for the recruitment of many secondary transcription factors, such as AP1, Sp1 or ATF, to some NF-κB target promoters [778]. This can also be the case for p65 bound to distal regulatory regions via

communication through looping of DNA. It is likely that p65 achieves the recruitment of secondary TFs to NF-κB target genes by directed chromatin alterations, such as histone acetylation [753]. Considering the functional diversity of NF-κB target genes the specific recruitment of secondary transcription factors could be one mechanism to allow a fine-tuning of the response on selected target genes. Additionally, it could prolong transcription by other factors after the IκB mediated removal of p65 from the promoter. As Wehkamp and colleagues mutated alternatively the two NF-κB and the one AP1 binding sites in the DEFB2 promoter in IECs they showed that the binding of AP1 is not sufficient for gene transcription in the absence of NF-κB binding. We can speculate that the binding of p65 is also necessary in our case for the recruitment of secondary TFs such as AP1 and that they work in synergy to increase the DEFB2 expression upon HDACi treatment.

VI.7 HDAC inhibitors as therapeutic options for inflammatory and infectious diseases

HDACs are important regulators of inflammation and innate as well as adaptive immunity [644]. They play key roles in TLR and IFN signaling pathways, regulation of macrophage and DC activity, antigen presentation, Th cell polarization and lymphocyte development and function. Interestingly, they can act as positive or negative regulators of TLR signaling, which is reflected in the opposing outcomes of HDAC inhibition on downstream target gene expression [779,780]. Both HATs and HDACs are implicated in inflammatory diseases. For example, asthma and COPD are associated with decreased HDAC expression and activity [781]. In COPD patients, H4 acetylation of IL8 promoter is increased, indicating a role of overexpression of this inflammatory cytokine in the pathology [782]. Diabetes on the other hand is linked to over activity of the HAT p300 stimulated by glucose leading to increased histone H3 acetylation at TNFα and COX2 promoters [783,784]. Few HDAC inhibitors are already used as anti-cancer agents and many more are in clinical trials for this purpose. Animal studies show potential for the use in treatment of inflammatory disorders but this new development of treatment is just starting [785].

The importance for histone acetylation in infectious diseases is underlined by the fact that several pathogens are able to induce specific deacetylation of target genes involved in the immune response due to yet unknown underlying mechanisms. *Mycobacterium tuberculosis* for example is able to induce histone deacetylation at the promoters of genes involved in antigen presentation and thereby repress their transcription [786]. The intracellular pathogen *Anaplasma phagocytophilum* was directly shown to activate the expression of genes encoding HDAC1 and HDAC2, which correlates with the decrease in H3 acetylation levels at the promoter of key immunity genes and their transcriptional repression [787].

To assess the therapeutic potential of HDAC inhibitors in the treatment of diseases with an inflammatory or infectious component it is important to understand the underlying regulatory mechanism of their actions. Additionally, the understanding of the effects of natural dietary HDAC inhibitors, such as butyrate, can help to decipher their role in the maintenance of homeostasis and health.

VI.7.1 The role of HDACs in the expression of inflammatory genes

Several studies have shown the anti-inflammatory properties of HDAC inhibitors. Leoni and colleagues for example found that suberoylanilide hydroxamic acid (SAHA) suppresses the production of pro-inflammatory cytokines TNFα, IL1β, IL12, IL6 and IFNγ induced by LPS stimulation *in vitro*, in human peripheral blood mononuclear cells and *in vivo*, in a mouse model [688]. They later repeated these results with another synthetic HDAC inhibitor, ITF2357 [689]. Two subsequent papers by Glauben and colleagues took these observations further into pathological conditions and linked the histone acetylation state to experimental colitis. They used the HDACis valproic acid (VPA) and SAHA to study their effect in dextran sulfate sodium- and trinitrobenzene sulfonic acid-induced colitis in mice, where oral administration of both drugs decreased disease severity [690]. This went hand in hand with a dose-dependent suppression of proinflammatory cytokines *in vivo*, induction of apoptosis in lamina propria lymphocytes, as well as a local increase in histone H3 acetylation. Again, these results were reproduced with ITF2357, where they Additionally, showed the prevention of inflammation-induced tumourigenesis by administration of the HDAC inhibitor [691].

More remarkably was the finding that HDAC inhibitors are not generally suppressing a pro-inflammatory response, but are able to regulate subsets of genes differentially. Leoni and colleagues for example did not find a suppression of IL8 by HDACi upon LPS stimulation [688]. Brogdon and colleagues treated dendritic cells and macrophages with the HDAC inhibitor LAQ824 before stimulation with LPS. The inhibitor alone induced both up and downregulation of genes in non stimulated macrophages, but selectively reduced or inhibited only a subset of LPS upregulated genes [780]. Specifically, it inhibited DC-controlled Th1 activation but not Th2 activation and migration. Furthermore, macrophage- and DC-mediated monocyte chemotaxis was inhibited, but not neutrophil chemotaxis. These results indicates that HDACs play differential roles in the regulation of expression of selected target genes and even whole physiological programs, and more understanding of their actions is needed to assess targeted treatment options.

VI.7.1 The role of HDACs in the expression of AMP genes

Very little has been described about the role of single HDAC enzymes in the expression of specific genes. Interestingly, one of the few studies describes the role of HDAC1 in AMP expression in the lung. Both, DEFB1 polymorphisms [435,436] and HDAC deregulation [782] are associated with the pathogenesis of COPD, where a chronic inflammation contributes to pathology. Treatment of lung epithelial cell lines with the HDAC inhibitor TSA for 24 hours increased DEFB1 expression 2-fold. More specifically, siRNA experiments showed an increase in DEFB1 expression upon knockdown of HDAC1. ChIP experiments could show an increase in acetylation of H3 and the transcription-promoting histone mark H3K4-me3 at the DEFB1 promoter upon specific HDAC1 inhibitor treatment [646]. The connection between DEFB1 expression and HDAC1 in the lung indicates a direct link between epigenetic regulation of AMP expression and disease. Moreover, this study shows the involvement of HDACs in AMP gene expression, as described in our model of IECs.

VI.7.1 The role of natural HDAC inhibitors in intestinal homeostasis

It is interesting to speculate about the role of natural HDACis, such as butyrate, in the maintenance of AMP expression in the intestine. *In vitro* studies in human colonic cell lines as well as *in vivo* studies in the rabbit model identified butyrate as an inducer of cathelicidin expression [264,534]. Additionally, treatment with the butyrate and another

dietary HDACi, sulforaphane, stimulated DEFB2 expression in colonic epithelial cells [538]. Besides, the role of SCFA in AMP stimulation, their influence on the local immune surveillance in the intestine is just being unraveled. Recent studies discuss directly their function as HDAC inhibitors in the maintenance of homeostasis. Chang and colleagues describe that treatment with butyrate *in vitro* or oral butyrate administration *in vivo* downregulated expression of proinflammatory cytokines, such as IL6, IL12 and nitric oxide by intestinal lamina propria macrophages [788]. This effect was attributed directly to the HDAC inhibiting activity in concordance with the effects of TSA. They suggest that this mechanism of rendering lamina propria macrophages hyporesponsive via commensal produced butyrate could play an important role in homeostasis maintenance in the gut. This hypothesis is supported by a study coming from Arpaia and colleagues, who showed that microbiota-generated SCFAs promote the differentiation of peripheral Tregs *in vitro* and *in vivo* and the accumulation of Tregs in the colon [751]. Comparison with the HDACi TSA lead them to the conclusion that those effects rely on the HDAC inhibiting capacity of butyrate and propionate. ChIP analysis of butyrate treated T cells showed a marked increase in H3K27 acetylation on the Foxp3 promoter and the intronic enhancer CNS1 (conserved non-coding sequence 1), but the mRNA level was not influenced. Instead, the protein concentration of Foxp3 was increased, which could be due to acetylation-mediated increase in stability of this factor [789]. Furthermore, butyrate primed DCs for the increased induction of Treg development. This activity was also correlated with the HDACi function. A comparison of TSA and butyrate treated DCs showed remarkably similar gene expression profiles, with a systemic repression of LPS response genes and the DC activity-regulating gene Relb. Others had shown before that knockdown of Relb promotes the ability of DCs to differentiate Tregs [790]. Together, a combination of differential DC activation, increased Foxp3 stability, and acetylation on the gene level of Foxp3 by HDAC inhibition can be envisioned to play a role in Treg generation by SCFA.

VI.8 Biological reasoning for the epigenetic regulation of DEFB2 expression

The question about the biological relevance of these regulatory mechanisms for AMP genes is intriguing. The requirement for stimulus-induced recruitment of transcription and co-factors, establishment of histone marks and chromatin remodeling before expression of a gene allows for a highly selective activation. Considering the versatile roles of DEFB2 in defense, but also in recruitment of immune cells, it would make sense to have the expression and inducibility of this gene tightly controlled. Moreover, it can be envisioned that the epigenetic control seen by acetylation in our model, mediates a gradual inducibility and expression of the DEFB2 gene, which can be adjusted according to the situation. In the basal state of homeostasis in the intestine, the DEFB2 gene remains unexpressed and other constitutively expressed AMPs regulate the interaction with gut commensals. In the case of a barrier breach DEFB2 expression is activated dependent on the inducing signal. This activation could possibly be fine-tuned and enhanced by epigenetic mechanism according to the type of commensal or pathogen, the amount of bacteria and the grade and duration of inflammation. In this scenario, it would make sense to enhance the antimicrobial response separated from the inflammatory response, which eventually needs to be shut down to avoid detrimental effects on the tissue. While a short period of expression can be enough for certain chemokines and cytokines to fulfill their role in recruitment and activation of immune cells, the expression of AMPs needs to be enhances and prolonged in certain situations, as they directly kill invading pathogens.

VI.9 Therapeutic perspective

If we consider the findings concerning the anti-inflammatory capacity of HDAC inhibitors and combine them with our findings on their boosting activity on AMPs and restitution gene expression, they seem to be promising candidates for the treatment of inflammatory conditions, where they at the same time could improve the antimicrobial defense and tissue repair. Further research will have to be undertaken to unravel the target gene specificity and enzyme specific effects on host physiology to better understand their mode of action and develop targeted treatment options.

The fact that many of the above mentioned substances are already used in cancer trials or even approved by the FDA is of advantage for the exploration of their therapeutic potential.

Chapter VII – Conclusion

Considering the possibilities for the regulation of inducible gene expression discussed above and the specific role of acetylation in the expression of DEFB2 in our model, we can imagine several scenarios by which pre-treatment with HDACi increases the kinetics and magnitude of its expression:

Regarding the role of HDACs and their inhibition by TSA we can imagine that the DEFB2 promoter is occupied with repressive HDAC-containing complexes in unstimulated cells. HDACi pre-treatment removes those complexes and primes the promoter for enhances expression.

A second point in this regard is the possible release of nuclear p65 from inhibiting HDAC1/2 complexes as well as the increased and prolonged nuclear location and facilitated promoter binding of acetylated p65 by HDAC inhibition. The role of nuclear p50 released from HDAC1 in transcription also has to be considered.

Concerning the chromatin state at the DEFB2 promoter it is possible, that HDACi treatment increases acetylation of the site at specific residues, which on one hand open up the structure and on the other hand recruit important coactivators and the transcription machinery.

Furthermore, the same can be expected for a possible enhancer region, which becomes accessible upon HDACi treatment and increases the transcriptional activity and output of the DEFB2 promoter.

As for the role of p300, we can imagine its action on two sites, which are important for DEFB2 expression: For once due to the acetylation of p65 and on the other hand due to acetylation of the promoter and possible enhancer region.

Interesting is the role of H3S10-P, which is highly increased upon HDACi treatment. Whether this mark is phosphorylated via the activation of MAPK pathways or IKK is still an open question. Its role in recruitment of p300 and downstream acetylation of H3K9 and H3K14 as well as in transcriptional elongation could be important for the enhanced the DEFB2 expression upon HDACi treatment.

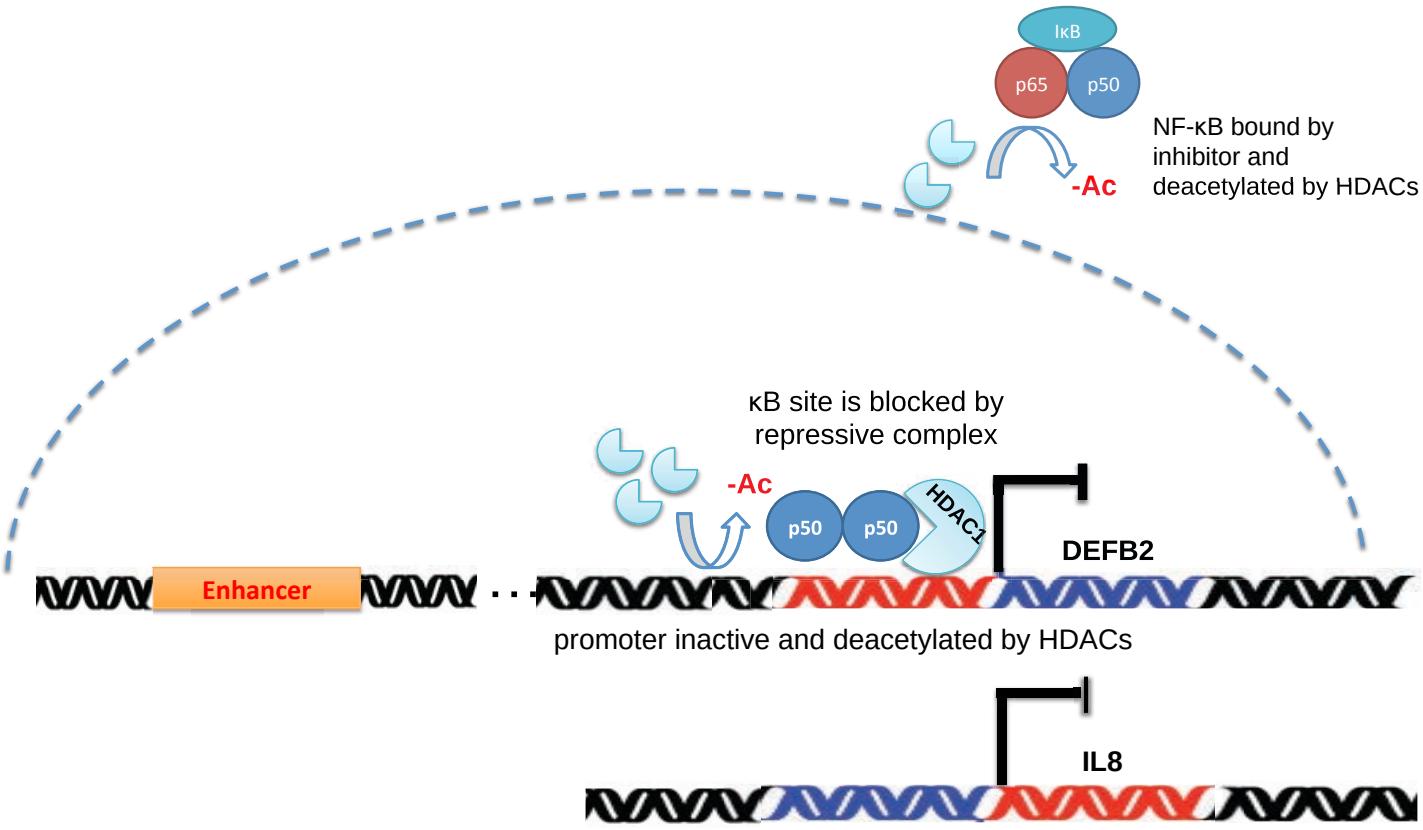
In regards of MAPK activation, we have to consider a possible role for AP1, as well as synergistic action with NF-κB.

The question of what distinguished the acetylation-dependent regulation of DEFB2 from the activation of IL8 is unsolved for now, but could be answered by additional ChIP experiments. It is likely that it is the result of targeted promoter or enhancer specific recruitment of cofactors, possibly via epigenetic modifications, which positively influence the DEFB2 expression but are absent in IL8 expression.

Chapter VIII – Model

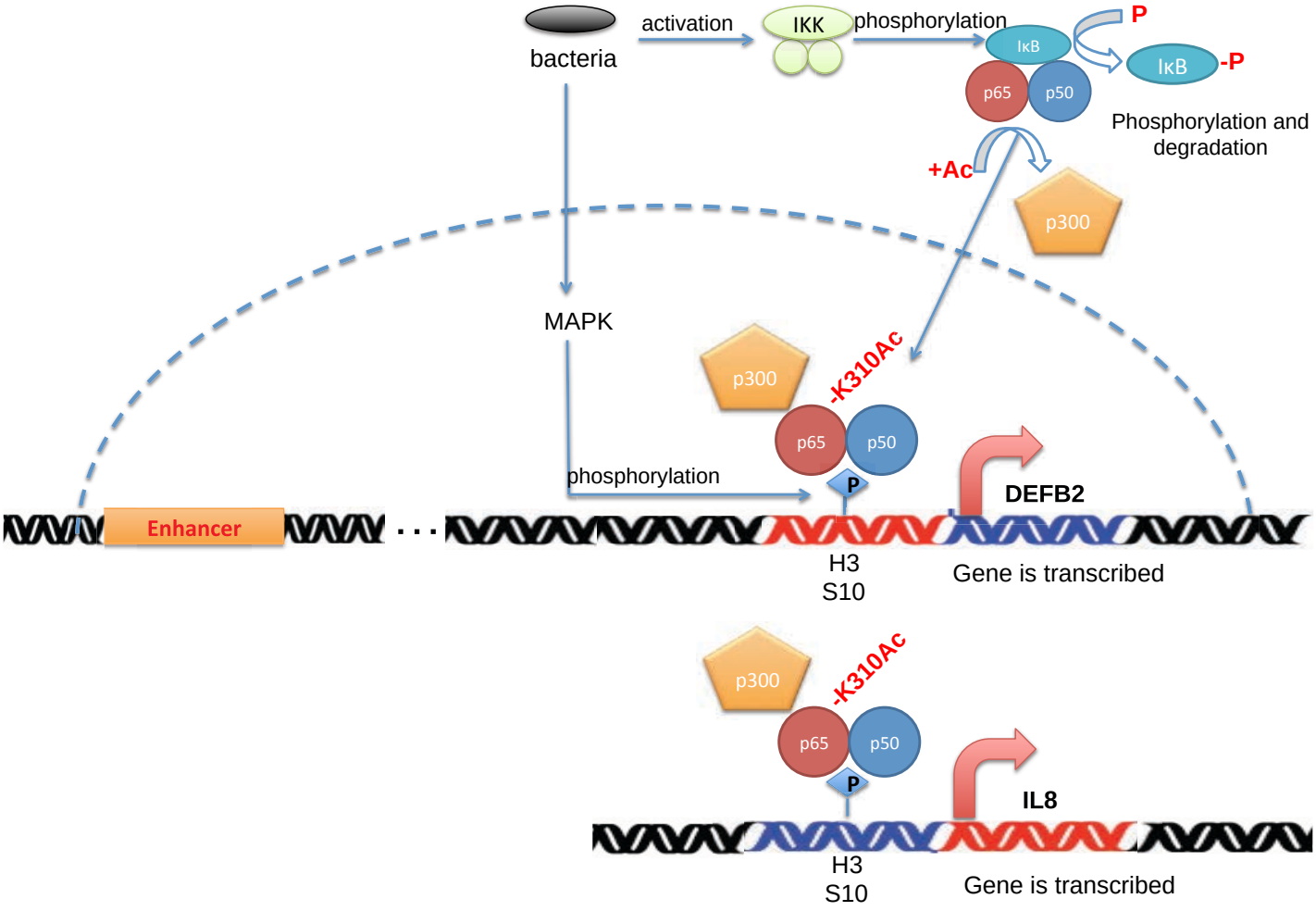
The presented results and discussion lead to the following models describing the influence of TSA on unstimulated cells and the activity of DEFB2 and IL8 gene expression in the circumstance of TSA treatment with and without bacterial challenge.

1. Unstimulated cell



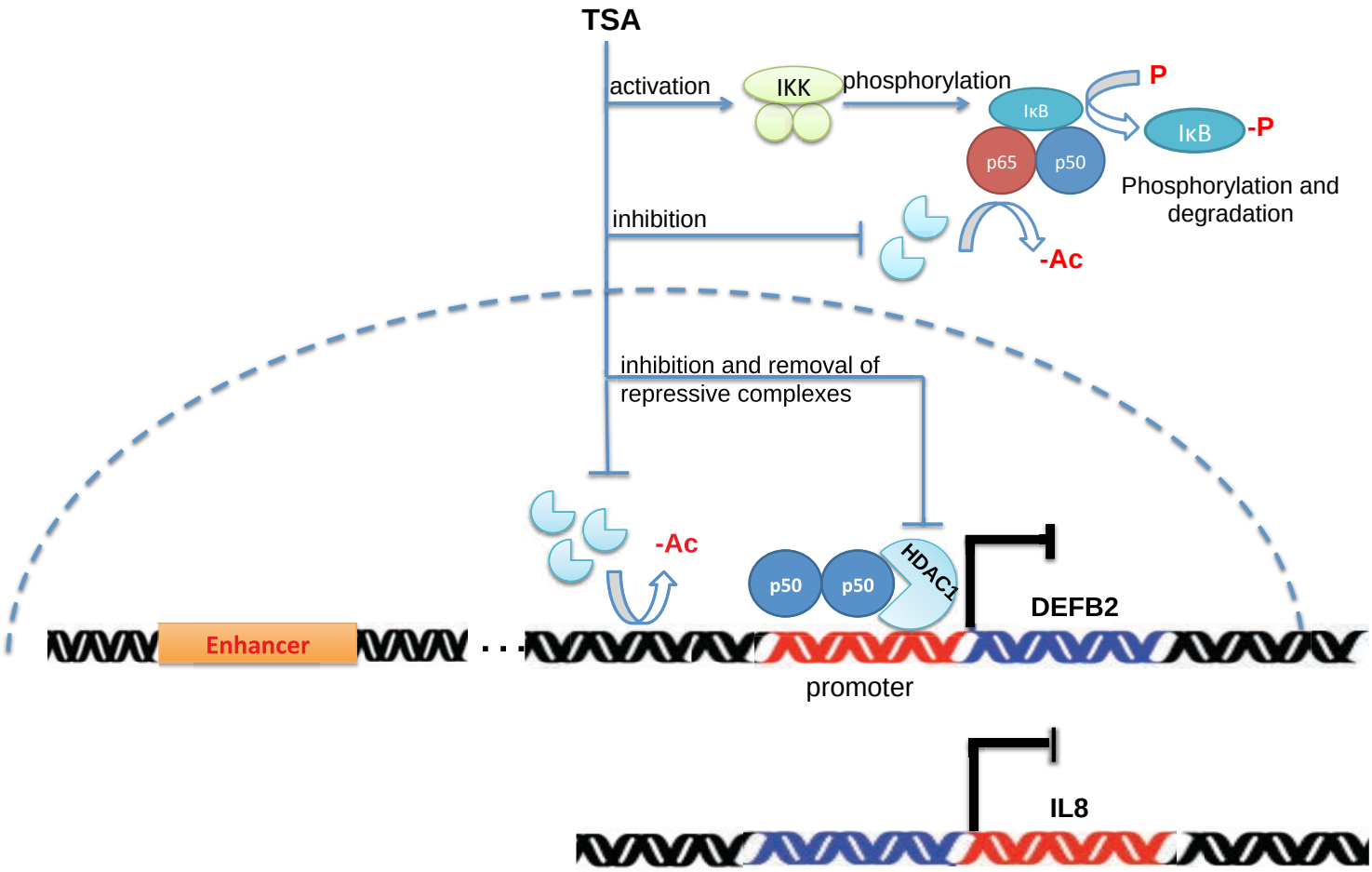
In unstimulated cells, the DEFB2 and IL8 promoter are inactive. We speculate that repressive complexes of p50/HDAC1 block the κB site at the DEFB2 promoter. Furthermore, the chromatin environment is maintained in a deacetylated state by the activity of HDACs. NF-κB is kept in the cytosol by its inhibitor and is kept deacetylated by HDAC enzymes.

2. Bacterial challenge



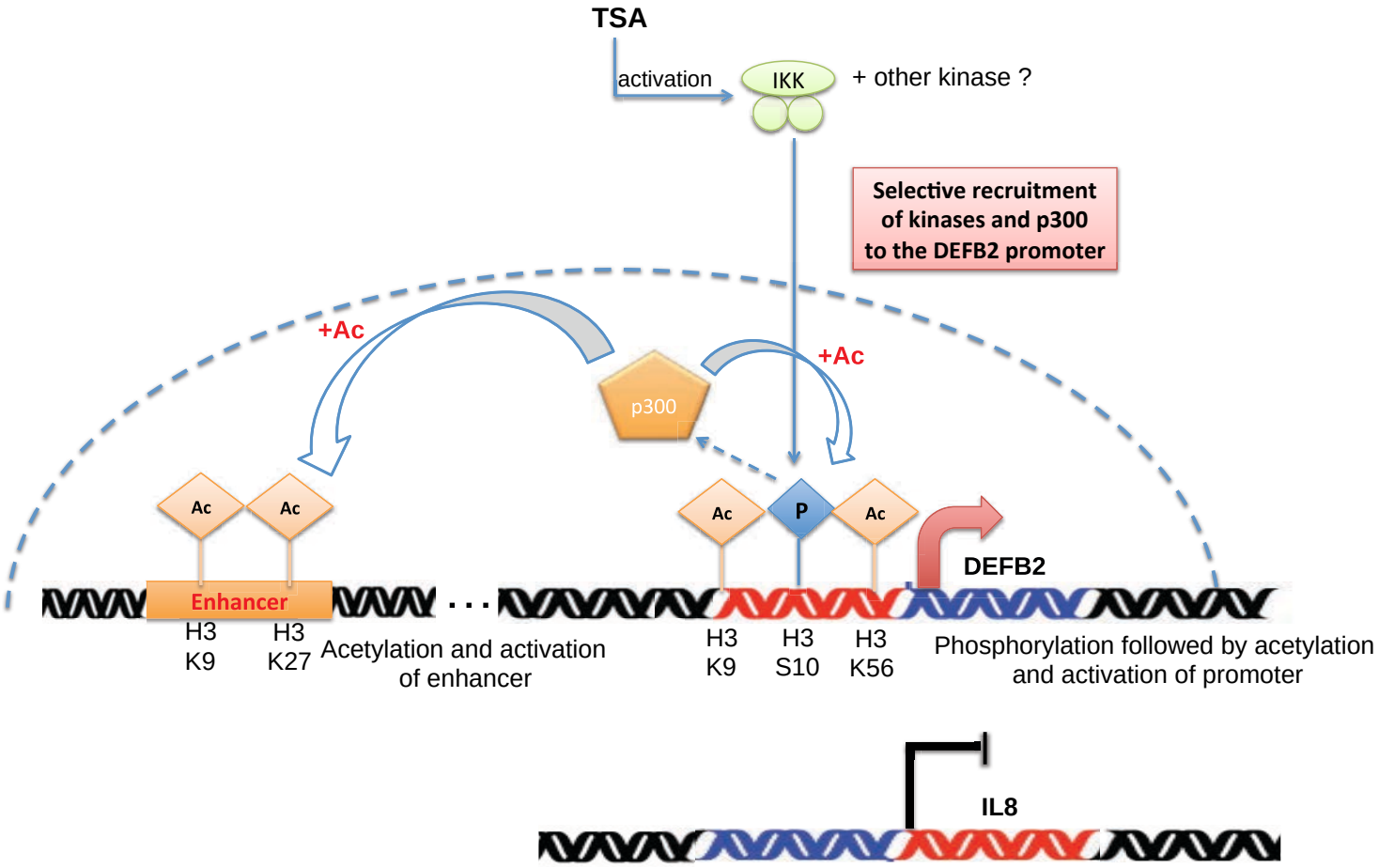
Upon bacterial challenge, several signaling pathways are activated. For once IKK complex phosphorylated IκB for degradation and thereby releases activated NF-κB, which is acetylated by p300. Additionally, MAPKs are activated and moderately phosphorylate H3S10, which leads to possible modifications of the chromatin environment and recruitment of cofactors, such as p300. Both the DEFB2 and IL8 promoter are activated for transcription by NF-κB and possibly other factors.

3.A Unstimulated cell + TSA



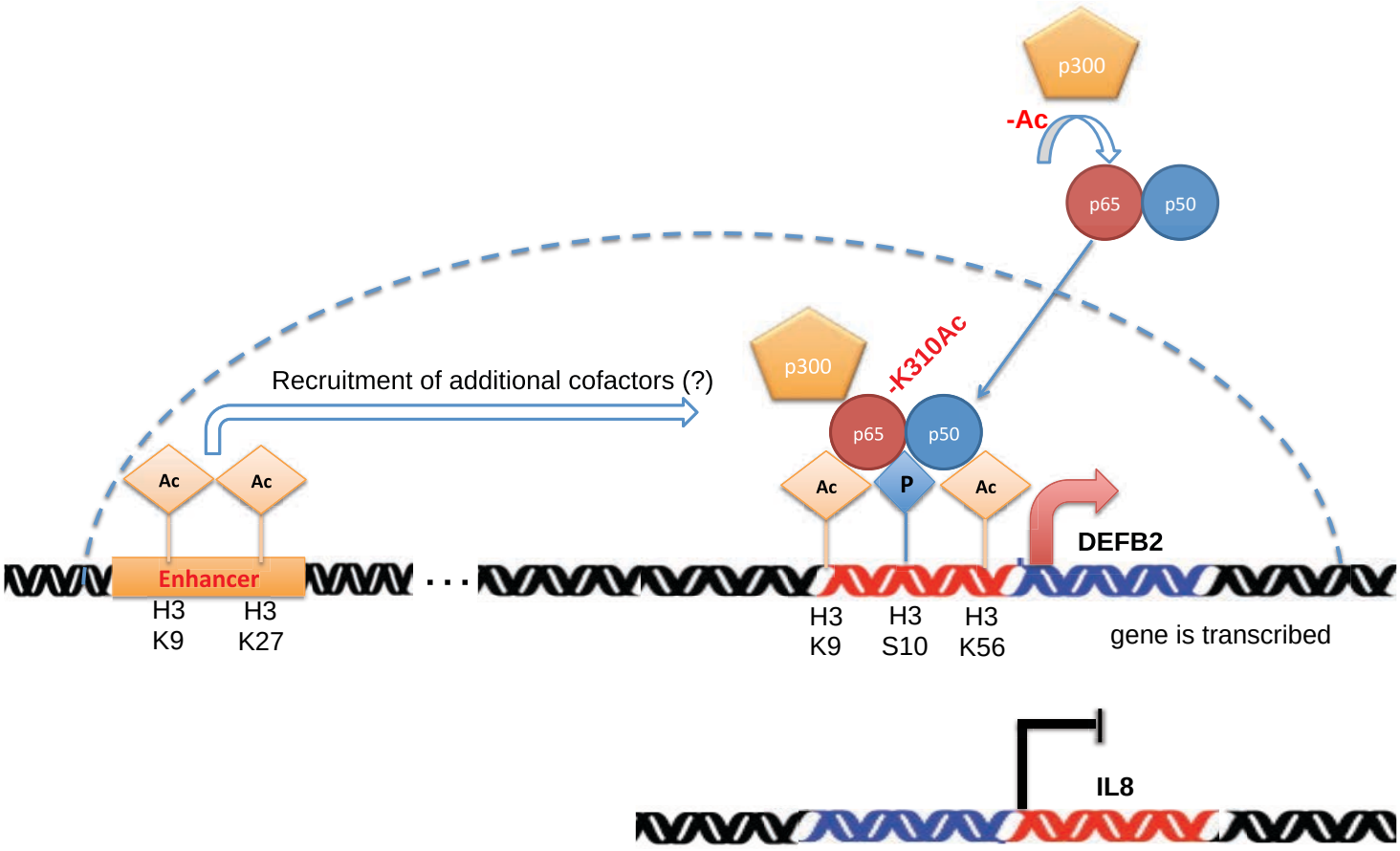
Upon TSA treatment of unstimulated cells, all classical HDACs are inhibited. Repressive complexes are removed from the DEFB2 promoter. Acetylation of histones as well as p65 becomes possible. Furthermore, TSA activates the IKK complex to phosphorylate and degrade IκB and release NF-κB.

3.B Unstimulated cell + TSA



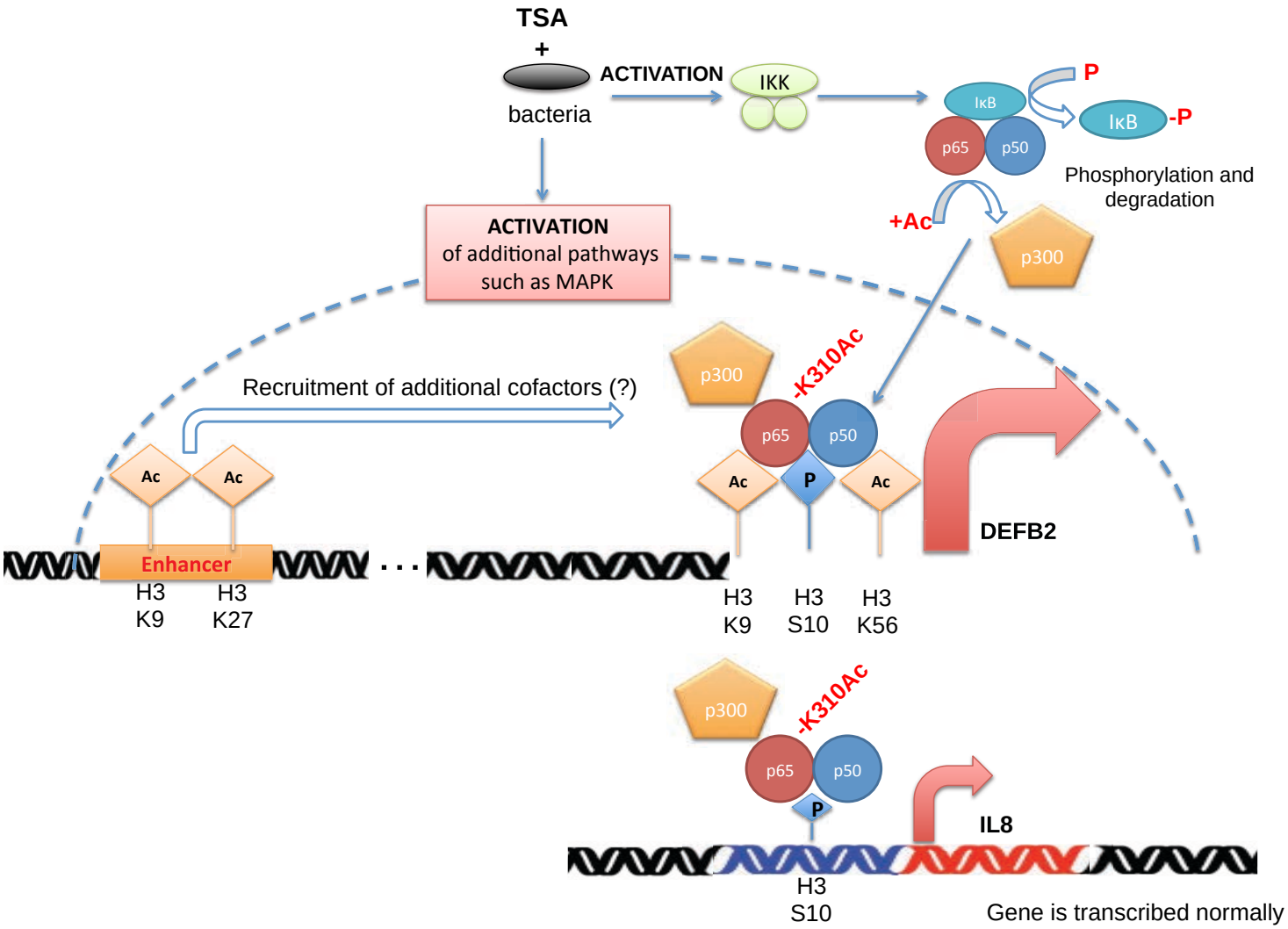
We hypothesize that TSA activates the IKK complex and possible other kinases, which are selectively recruited to the DEFB2 promoter and lead to a massive phosphorylation of H3S10-P. This mark recruits p300, which mediates *de novo* acetylation of H3K9, H3K27 and H3K56 specifically at the DEFB2 promoter. Furthermore, we suggest the existence of a distal enhancer element, which could be activated by p300, indicated by acetylation of H3K9 and H3K27.

3.C Unstimulated cell + TSA



We propose that the increase in basal DEFB2 expression upon TSA treatment in unchallenged cells is mediated by NF-κB, which is selectively recruited to the modified DEFB2 promoter, but not to the same extend to the IL8 promoter in this case. Furthermore, it is possible that the activated enhancer facilitates the recruitment of additional cofactors and transcription factors.

4. TSA followed by bacterial challenge



Finally, in the case of bacterial challenge of TSA-pre-treated cells the activation of NF-κB and other signaling pathways is significantly enhanced. Together with the pre-setting of the DEFB2 promoter by TSA the bacterial signals lead to an enhanced expression of DEFB2, while IL8 is expressed at a normal level as seen upon infection.

Chapter IX – Experimental perspectives

The discussion of the presented results in a bigger picture raises many unsolved questions regarding the regulation of DEFB2 expression on epigenetic as well as genetic levels. It becomes more and more clear that these two levels of regulation as tightly intertwined and cannot be regarded separately. The interplay between factors of the transcription machinery and chromatin modifying enzymes is essential for inducible gene expression. The question if underlying programs for the recruitment of these factors exist in the DNA sequence is intriguing and cannot be answered straightforward at this moment.

As for the regulation of DEFB2 expression, in general ChIP experiments will have to be performed to examine the localization of factors such as p300, HDAC1/2, p65, p65K310-Ac, p50, AP1, Brd4, p-TEFb and Pol II as well as occurrence of specific histone marks H3S10-P, H3K9-Ac, H3K14-Ac, H3K27-Ac and H3K56-Ac in the basal state, upon HDACi pre-treatment and upon infection.

Comparison with the promoter of IL8, whose expression is not boosted by HDACi treatment in our setting, will give a possible negative control and allow assessment of DEFB2 specific regulatory mechanisms.

Additionally, the investigation of our observations in human *ex vivo* tissue would be an important validation of our findings.

Chapter X – Bibliography

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Annex

Antimicrobial peptides (AMPs) are ancient, conserved molecules of the innate immune system, expressed on all the bodies' epithelial surfaces in contact with the environment and its microbes. The intestine harbors a huge microbiological ecosystem, and AMPs secreted by the intestinal epithelium are at the forefront of homeostasis maintenance. They actively kill and disable a wide variety of bacteria, viruses, fungi and protozoa. Moreover, their immunomodulatory functions are important in the activation of the adaptive immune response and tissue regeneration. Their importance becomes clear, as their expression is correlated with the susceptibility to infection and deregulation of their expression has been associated with a multitude of severe human pathologies.

Some AMPs are expressed constitutively, others are inducible by a range of bacterial, nutritional and host-derived signals. The regulation of inducible AMP expression is widely undiscovered. The role of chromatin remodeling and histone modifications, as an additional regulatory level to transcription factor activation, in the expression of inducible genes is becoming more and more clear.

The aim of this work was to investigate the (epi)genetic mechanisms, which are involved in the regulation of AMPs gene expression in the intestine. By the use of specific inhibitors of chromatin modifying enzymes, in an *in vitro* model of intestinal epithelial cells challenged with *E. coli* strains, we discovered the importance of acetylation in the regulation of these genes. Inhibition of histone deacetylase enzymes (HDACs) significantly enhanced the bacteria-induced expression of the beta-defensin-2 (DEFB2) and other AMPs, while the expression of the interleukin 8 (IL8) and other inflammatory genes was not influenced. Furthermore, we detailed the molecular mechanism, especially involvement of the transcription factor NF-κB and the histone acetyltransferase p300 in this observation. This discovery presents a mechanism of (epi)genetic enhancement of AMPs expression, dissociated from the pro-inflammatory response.

Les peptides antimicrobiens sont des effecteurs de l'immunité innée produits par les cellules épithéliales en réponse à la présence microbienne. Au niveau de l'intestin, la sécrétion de ces peptides est directement impliquée dans les processus homéostatiques existant entre un hôte et son microbiote. Ces peptides exercent leur activité microbicide sur un large spectre de microorganismes, incluant les bactéries, les virus, les champignons et les protozoaires. Ils possèdent également des activités immuno-régulatrices impliquées dans l'activation de la réponse immunitaire adaptative et le processus de régénération tissulaire. Ces dernières années, plusieurs études menées chez l'homme ont démontré une corrélation entre le niveau d'expression des gènes codant les peptides antimicrobiens et la susceptibilité des individus à différentes pathologies.

Les peptides antimicrobiens peuvent être exprimés constitutivement ou de manière inductible en réponse à différents signaux de type bactériens, nutritionnels ou endogènes à l'hôte. Dans les deux cas, les mécanismes de régulation génétique et épigénétique mis en jeu par la cellule épithéliale intestinale pour contrôler leur expression sont méconnu.

Le but de ces travaux de thèse a été d'étudier la composante (épi)génétique des régulations contrôlant l'expression des gènes codant les peptides antimicrobiens. Utilisant un modèle de cellules épithéliales intestinales humaines exposées à différentes molécules inhibitrices de l'activité d'enzymes modifiant la chromatine, et stimulées par la bactérie *Escherichia co*li, nous avons identifié l'importance du processus d'acétylation dans le niveaux d'induction de l'expression des ces gènes. Nous avons montré que l'inhibition des enzymes de la famille des histone déacétyases (HDACs) augmente significativement le niveau d'induction des gènes antimicrobiens comme DEFB2 (béta-défensine-2), sans impacter celui des gènes pro-inflammatoires comme l'IL8 (interleukine 8). Enfin, nous avons étudié le mécanisme moléculaire sous-jacent à cette observation, notamment le rôle du facteur de transcription NF-κB et celui de l'enzyme p300 appartenant à la famille des histone acétyltransférases. Ces travaux démontrent l'existence d'un mécanisme (épi)génétique permettant de réguler différentiellement le niveau d'induction des gènes antimicrobiens et pro-inflammatoires.