IMMUNOMODULATION BY BACTERIOPHAGES

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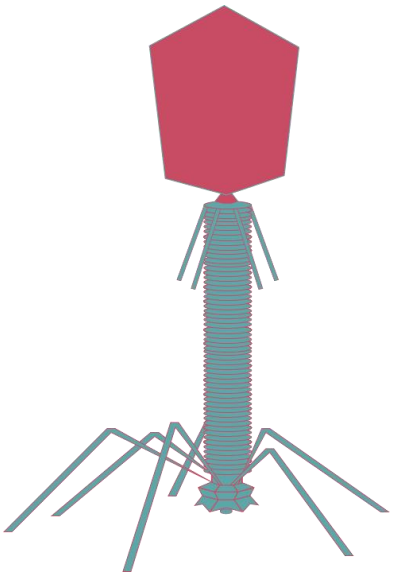

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Acknowledgments

A window into my mind.

As it is custom to write an acknowledgment when writing a PhD thesis, so shall I write one. I have to admit that this was one of the hardest parts to write not because I don't know who to thank, but more because I don't know who to thank first.

At the beginning of this PhD adventure I had no idea whether I would ever end up writing a PhD thesis, as I could not image gathering enough data to write one. During my PhD I learned that mens biggest friend and foe is its mind. It creates and destroys. Luckily for me, and don't mind me being a bit narcissistic, my mind is quite positive (perhaps brilliant?). It creates more than it can destroy, so in the end I was able to write this marvelous PhD thesis, if I say so myself.

But still I wonder, after these four years of science, can I call myself a scientist? Marie Skłodowska Curie once described a scientist as someone that not only knows his way around a laboratory, but as a child placed before a natural phenomenon which impresses him or her like a fairy tale. And that's exactly how I see science, as a world of magic and wonder. A place where much is known but even more is to be known, where the roads to discovery are adventures that are undertaken with much pleasure and enjoyment. Where the tales of others are inspirations for new discoveries or even new fields. In short it is amazing to be part of, it is like your own little Middle Earth or Westeros.

One of the first people I would like to thank are my supervisors, Mario and Rob. Without your continuing guidance I don't know whether I would have been able to finish this PhD, or whether I would have loved phages as much as I love them now. I would like to send my gratitude for your never-ending patients in my sometimes grand endeavors. Foremost I thank you for giving me the opportunity and encouraging me to broaden my mind by going to different international conferences

and seminars, where I was able to meet and talk to people who shared a common interest, phages. Where I could be the phage nerd that I am, where people shared my enthusiasm, and where I was able to meet some of those brilliant phage minds. Some of which I was able to turn into long time phage friends. Phage friends that have helped me stay critical about my research but also about phage research in general, who showed me the many possibilities of phages. The fact that I was able to attend these conferences especially these last two, undoubtedly shaped my future (phage?) research career. For this I thank you!

Maya, besides the fact that you might have been a time-traveler as you appear on photo's next to d'Herelle and Eliava, during these four PhD years you were always close by. Your inexhaustible phage knowledge has inspirited me from the start and it has been a true honor to learn many of the phage techniques directly from you. It brings me true happiness that you were willing to share your knowledge and that I can call you my friend. To you I say, never stop baking! Your cakes, pies and chocolates are stunning!

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This PhD thesis certainly would not have been possible without the help of CEVAC, more specifically Frédéric Clement. You gave us the initial idea of how to tackle the immune problem during my master dissertation. You gave us the opportunity to use your facilities, without which this dissertation was certainly doomed to fail. Well that might be a bit of an exaggeration, but still it gave us a nice advantage. Thanks also goes to Peter, Leen and Sabrina, together with their many colleagues of CEVAC who were always ready to help or give advice. I know that most of the time I was probably just keeping you from your work, but still you don't know how much I appreciated your help. Even when it seemed like it was no help at all.

Now it is probably time for the most personal section of this acknowledgement, the section where I thank my friends and family. I could start with thanking myself for constantly believing in myself, but that would be way to egocentric and a lie as well. If it weren't for the constant support of my parents and brothers (Marnix and Steven), I wouldn't know what I would have done. Mom, Dad, thank you for the uncountable opportunities you gave me. For always believing in me and for supporting all my wildest dreams. Thank you Marnix, Marijke, Nand, Ella and <…in progress, although Nand would have preferred a dog…> for the much needed distractions.

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Thank you Joe Yee for your constant support, you entered my life when the hardest part of my PhD had yet to start, the writing. You stuck with me during this harsh period and you have no idea how much that means. You are the wind beneath my wings. In the meanwhile we underwent a few adventures on our own, and hopefully you'll join me for our next big quest (whatever it may be).

Thank you Leen. Yes, yet another Leen. It seems as if the world only consists of Leens? Thanks for moving in when Steven left the apartment for his Puerto Rican adventures, leaving me with a way to expensive "kot". The late night talks that we had, or just the talks during dinner or TV always seemed to relax me to some extent. You often gave me the input that I needed in order to not see myself as some crazy scientist.

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talking about you! During those years you undoubtedly must have thought, what the frack is he talking about. To be honest, more than half of the time I myself had no clue what I was trying to say (but I'll still deny this in conversations). Still we had some pretty good times, hopefully you'll join me for some more!

In the end there are not enough words to express my gratitude to everyone who had a part in this thesis. I will never forget the many adventures I had these four years, and I know that this will probably be the only part that will be read by everyone holding this book (perhaps I should include some of my results in here?).

Let me end with the legendary words of Homer J Simpson:

> "The war has ended and the future won, the past never had any chance man!"
>
> The Simpsons - American History X-cellent (S21E17)

Until we meet again!

Jonas D Van Belleghem

13/12/2017 Ghent

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List of Abbreviations

A	
ABC	ATP-binding cassette
Abi	Abortive infection
AID	Activation-induced cytidine deaminase
AIRE	Autoimmune regulator
AMP	Adenosine monophosphate
APC	Antigen presenting cell
APE	Apurinic/apyrimidinic endonuclease
ASC	Apoptosis-associated speck-like protein containing a CARD
ATP	Adenosine triphosphate
B	
BAM	Bacteriophage adherence to mucus
BCR	B-cell receptor
BER	Base excision repair
BFGF	Basic fibroblast growth factor
BIR	Baculovirus inhibitor of apoptosis repeat
bp	Base pair
C	
C	Constant gene element
cAMP	Cyclic AMP
CAMP	Cationic antimicrobial peptide
CARD	Caspase activation and recruitment domain
Cas	CRISPR-associated
cDC	Conventional dendritic cell
CF	Cystic fibrosis
C_H	Immunoglobulin heavy chain constant
CIS	Cytokine-induced SRC-homology-2 protein
CLIP	Class II-associated Ii peptide
CMC	Critical micellar concentration
CNTF	Ciliary neurotrophic factor
CR1	Complement receptor 1
CR2	Complement receptor 2

CR3	Complement receptor 3
CRISPR	Clustered regulatory interspaced short palindromic repeat
CRP	C-reactive protein
crRNA	CRISPR RNA
CSR	Class switch recombination
CT1	Cardiotrophin-1
CTL	Cytosolic T lymphocyte
D	
D	Diverse gene element
DCs	Dendritic cells
DD	Death domain
DEG	Differentially expressed gene
DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
dsDNA	Double stranded deoxyribonucleic acid
dsRNA	Double stranded ribonucleic acid
dU	Deoxyuracil
E	
EF-2	Elongation factor-2
EGF	Epidermal growth factor
EPO	Erythropoietin
Erm	Erythromycin methylase
ER	Endoplasmatic reticulum
ESBL	Extended-spectrum ß-lactamase
ETA	Exfoliative toxin A
ETB	Exfoliative toxin B
EU	Endotoxin units
EU	European Union
F	
G	
GAP	GTPase-activating protein
G-CSF	Granulocyte colony-stimulating factor
GEO	Gene expression omnibus
GH	Growth hormone

GM-CSF	Granulocyte-macrophage colony-stimulating factor
GO	Gene ontology
GPCR	G-protein-coupled-receptor
GPI	Glycophosphatidylinositol
H	
HAP	Hospital-acquired pneumonia
HCAP	Healthcare-associated pneumonia
HCMV	Human cytomegalovirus
Hep	L-glycero-D-manno-heptose (Hep)
HGT	Horizontal gene transfer
HMG1	High-motility-group 1
HMG2	High-motility-group 2
Hoc	Highly antigenic outer capsid protein
HSV1	Herpes simplex virus 1
I	
iE-DAP	γ-D-glutamyl-meso-diaminopimelic acid
IFN	Interferon
Ig	Immunoglobulin
IGF1	Insulin-like growth factor-1
Ii	Nonpolymorphic invariant chain
IKK	IκB kinase
IL	Interleukin
IL1R	Interleuking 1 receptor
IL1RN	IL1 receptor antagonist
IRAK-4	IL1 receptor associated kinase-4
ISGs	Interferon-stimulated genes
ITAM	Immunoreceptor tyrosine-based activation motif
J	
J	Joining gene element
JAK	Janus kinase
K	
Kdo	3-deoxy-D-manno-octulosonic acid
KGD	Lys-Gly-Asp
L	

LBP	LPS-binding protein
LIF	Leukemia inhibitory factor
LPS	Lipopolysaccharide
LRR	Leucine-rich-repeat
LAT	Linker of activated T cells
LTA	Lipoteichoic acid
M	
MAC	Membrane attack complex
MAL	TIRAP/MyD88-adaptor like
MAPK	Mitogen-activated protein kinase
MASP 1	MBL-associated serine protease 1
MASP 2	MBL-associated serine protease 2
MASP 3	MBL-associated serine protease 3
MBL	Mannose-binding lectin
M-CSF	Macrophage colony-stimulating factor
MDP	Muramyl dipeptide
MFS	Major facilitator subfamily
MHC	Major-histocompatibility-complex
MIIC	MHC class II compartment
MIP-1α	Macrophage inflammatory protein 1α
MMR	Mismatch repair
MMTV	Mouse mammary tumor virus
MPS	Mononuclear phagocyte system
MSCRAMMs	Microbial surface components recognizing adhesive matrix molecules
Myd88	Myeloid differentiation primary response gene 88
N	
NHEJ	Nonhomologous end-joining
NLR	NOD-like receptor
NOD	Nucleotide binding oligomerization domain
O	
OM	Outer membrane
OSM	Oncostatin M
P	
PAMP	Pathogen-associated molecular patterns

PBP	Penicillin binding protein
PBP2a	Penicillin-binding protein 2a
PCNA	Processivity factor proliferating cell nuclear antigen
pDC	Plasmacytoid dendritic cell
PDGF	Platelet-derived growth factor
PG	Peptidoglycan
PLCγ1	Phospholipase Cγ1
PMN	Polymorphonuclear lymphocytes
Pol ß	DNA polymerase ß
PRL	Prolactin
PRR	Pattern recognition receptors
PTSAg	Pyrogenic toxin superantigen
Q	
QRDR	Quinolone resistance-determining region
R	
RAG1	Recombination-activating gene 1
RAG2	Recombination-activating gene 2
RD	Repressor domain
rFC	Recombinant factor C
RHIM	Rip homotypic interaction motif
RIP1	Receptor-interacting protein 1
RLR	RIG-I-like receptor
R-M	Restriction-modification
RNA	Ribonucleic acid
RND	Resistance/nodulation/cell division family
RORγt	Retinoic acid receptor related orphan receptor γt
ROS	Reactive oxygen species
RSS	Recombination signal sequence
RSV	Respiratory syncytial virus
S	
SARM	Sterile α and HEAT-Armadillo motifs-containing protein μ
SEA	Staphylococcal enterotoxin A
SEB	Staphylococcal enterotoxin B
SEC	Staphylococcal enterotoxin C
SED	Staphylococcal enterotoxin D

SEE	Staphylococcal enterotoxin E
SEG	Staphylococcal enterotoxin G
SEH	Staphylococcal enterotoxin H
SEI	Staphylococcal enterotoxin I
SH2P	SH2-domain-containing protein tyrosine phosphatase 2
SHM	Somatic hyper mutations
sMAP	Small MBL-associated protein
SMR	Small multidrug regulator family
SOAP	Short oligonucleotide analysis package
SOAP2	SOAP aligner
SOCS	Suppressor of cytokine signaling
SSB	Single stranded DNA break
ssDNA	Single stranded deoxyribonucleic acid
ssRNA	Single stranded ribonucleic acid
STAT	Signal transducer and activator of transcription

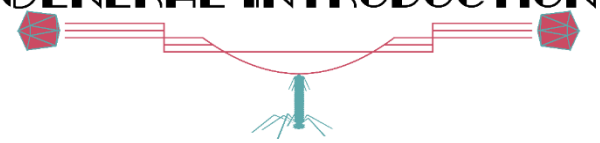
T

T2SS	Type 2 secretion system
T3SS	Type 3 secretion system
TAK1	Transforming growth factor-ß-activated kinase
TANK	TRAF family member-associated NF-κB activator
TAP	Transporter associated with antigen presentation
TBK1	TANK binding kinase 1
TCR	T-cell receptor
TdT	Terminal deoxynucleotidyl transferase
TGFß	Transforming growth factor-ß
TICAM1	TRIF/TIR-domain-containing molecule 1
TIR domain	Toll/IL1R homology domain
TIRAP	TIR-associated protein
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TPO	Thrombopoietin
TRAF6	TNF receptor-associated factor 6
TRAM	TRIG-related adaptor molecule
TRIF	TIR-domain-containing adaptor protein-inducing IFN-ß
TSH	Thyrotropin

TSLP	Thymic stromal lymphopoietin
TSST-1	Toxic shock syndrome toxin-1
U	
UBC13	Ubiquitin-conjugating enzyme 13
UDP-GlcNAc	UDP-*N*-acetylglucosamine
UEV1A	Ubiquitin-conjugating enzyme E2 variant 1 isoform A
V	
V	Variable gene element
VAP	Ventilator-associated pneumonia
VISA	Vancomycin intermediate-resistance *Staphylococcus aureus*
W	
WHO	World Health Organization
X	
Y	
Z	

Chapter 1

General Introduction



"Whatever it is you're seeking won't come in the form you're expecting."

Kafka on the Shore – Haruki Murakami

Bacteria that inhabit the intestine and skin are generally regarded as stable residents that may confer metabolic and/or immune benefits to their hosts (Turnbaugh et al., 2009). The host immune system has evolved mechanisms to tolerate these commensal organisms while at the same time providing protection of the host from pathogens (Moon and Stappenbeck, 2012). Similarly, metagenomic studies of microbiota at various tissue sites have revealed that a vast variety of bacteriophages (phages) are associated with healthy human tissues (Minot et al., 2011; Pride et al., 2012; Reyes et al., 2010). In the case of phages, a persistent nonpathogenic association seems possible as viral replication occurs in bacterial hosts, which can themselves be stable members of the microbiota (Duerkop and Hooper, 2013).

It is known that the oral administration of phages leads to the translocation of the phages from the gut to the blood (Duerr et al., 2004; Hamzeh-Mivehroud et al., 2008; Weber-Dąbrowska et al., 1987). This suggests that mammals have evolved mechanisms for the uptake and delivery of phages that may allow intestinal phages to elicit innate and adaptive immune responses. Knowing that phages are present everywhere and that we are in constant contact with phages, the question can be asked to what extent phages interact with our immune system. More specifically, do they have anti-inflammatory properties? Otherwise, how can they work systemically without inducing an immune response?

In order to find an answer to these questions it is not only important to have a broader understanding of the human immune response but also to understand the need of phage therapy when antibiotic resistant bacteria are becoming an increasing problem, and why it is important to investigate the interaction of phages with the human immune system. **Chapter 2- Immunity** gives a detailed account of the human immune response, which can be divided into two branches: the innate and the acquired immune response (Akira et al., 2006). The main function of the *innate immune response* is to provide a direct defense against invading pathogens. These defenses are mainly provided by recognizing pathogens by means of pathogen-associated molecular patterns (PAMPs) (Janeway, 1989). This innate immunity is responsible for most of the inflammatory responses and are triggered in first instance by macrophages, polymorphonuclear leukocytes and mast cells (Janeway and Medzhitov, 2002). The recognition of PAMPs is mediated through pathogen recognition receptors (PRRs), such as Toll-like receptors (TLR), which activate signal-transduction pathways that induce the expression of a variety of immune-response genes (Kawai and Akira, 2010; Kumar et al., 2009a; Ozinsky et al., 2000; Takeuchi and Akira, 2010).

The *adaptive immunity* is activated upon the recognition of a pathogen by antigen presenting cells (APCs) (Pulendran et al., 2001). These dendritic cells phagocytose the pathogen and subsequently undergo cell maturation. This includes the induction of costimulatory activity, antigen processing, increased major histocompatibility complex (MHC) molecule expansion, and migration into the

lymph nodes (Orsini et al., 2003). The adaptive immune system manifests an exquisite specificity for its target antigens, unlike the innate immunity. The so-called T and B cells play major key roles in the adaptive immune response, through the expression of specific receptors (Bonilla and Oettgen, 2010; Chaplin, 2010; Schroeder and Cavacini, 2010).

Regarding the reduction of human morbidity and mortality, the introduction of antibiotics was one of the most important medical interventions. Unfortunately, the intense (mis)use of antibiotics has led to an increased frequency of antibiotic resistant bacteria (Guay, 2008; Lew et al., 2008; Woodford and Livermore, 2009). Chapter 3 – Antibiotics discusses the mechanisms by which antibiotic resistance might occur. By understanding these antibiotic resistance mechanisms, it becomes clear why alternative strategies for combating multidrug resistant bacteria such as phage therapy are promising new therapies.

Chapter 4 – Endotoxins gives a complete description about the function and biological characteristics of lipopolysaccharides. Endotoxins (lipopolysaccharides, LPS) produced by Gram-negative bacteria are widely recognized for their immunological properties, as they cause septic shock, multiple organ dysfunction and failure (Epstein and Parrillo, 1993; Petsch and Anspach, 2000). Therefore, it is important to understand their chemical structure and biochemical properties, not only to understand the mechanisms by which they are made or interact with the human immune system, but also to develop strategies to circumvent their pro-inflammatory properties or develop protocols for their removal from biological samples. The removal of endotoxins from phage preparations becomes important when using phages that have Gram-negative bacteria as their host.

Chapter 5 – Bacteriophages provides an in-depth description of these bacterial viruses, also called phages. The emergence of pathogenic bacteria resistant to most, if not all, currently available antibiotics has become a critical problem in modern medicine. Prior to the discovery and widespread use of antibiotics (around World War II), it had been suggested (around World War I) that bacterial infections could be prevented and/or treated by the administration of bacteriophages. Bacteriophages are the most abundant entities on Earth with an estimated number of 10^{30} particles (Suttle, 2005). Every bacterial cell can harbor many phages. These viruses use bacteria as a mean to replicate, invariably destroying their prokaryotic host in the process. Current knowledge states that phages ignore every cell except the strain of bacteria they have evolved to infect. This makes them ideal candidates to treat bacterial infections, while being harmless to mammalian cells and even non-target bacteria (Thiel, 2004).

Additionally, it has been demonstrated that oral uptake of phages by animals results in the translocation of phages to systemic tissues (Duerr et al., 2004; Hamzeh-Mivehroud et al., 2008). This suggests that mammals have mechanisms

for the uptake and delivery of phages. The resulting contact between systemic tissues and phages may allow phages to elicit innate and adaptive immune responses. One possible uptake route involves dendritic cells, which are known to sample intestinal luminal contents and can actively phagocytize phage particles in culture (Barfoot et al., 1989; Rescigno et al., 2001). It is also interesting to consider whether phages might elicit antiviral innate immune responses. Mammalian cells are endowed with the ability to detect eukaryotic viral nucleic acids through several pattern-recognition receptors that are positioned to detect viral entry into cells (Duerkop and Hooper, 2013). For a global overview of phage-human host interactions see Chapter 5.5.

Finally, in order to answer the question stated above: *"do bacteriophages have immunological properties"*, this dissertation can be subdivided into two main parts:

Endotoxin removal strategies.

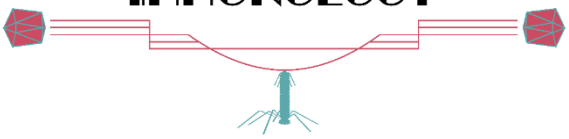
The presence of endotoxins in samples, whether they are proteins, pharmaceuticals or phages play an important role in the outcome of their observed effects. Therefore, it was important to use highly purified, endotoxin-free, phage preparations to deduce whether phages are able to induce an immune response. Seven different endotoxin removal strategies were evaluated and validated for their endotoxin removal efficacy. The results obtained regarding these seven endotoxin removal strategies are discussed in Chapter 6 – Endotoxin purification strategies and have been published (Van Belleghem et al., 2017a).

Bacteriophage induced immune responses.

Once highly purified phage preparations were obtained, their potential to induce an immune response was evaluated. This was done by stimulating peripheral blood mononuclear cells (PBMCs) with these highly purified phage preparations, after which the gene expression of twelve immunity-related genes was evaluated. The results concerning these immune responses are described in Chapter 7 – Transcriptome analysis of phage stimulated PBMCs, Chapter 8 – Immunomodulation by bacteriophages, and Chapter 9 – *In silico* modeling: the phage – bacteria – innate immune response interaction. The combined results of these first two chapters are summarized and published in Van Belleghem et al. (2017b).

Chapter 2

IMMUNOLOGY



"Somewhere, something incredible is waiting to be known."

Carl Sagan

Introduction - Human immune response

Living organisms are constantly threatened by the invasion of microorganisms and vertebrates have evolved elaborate systems of immune defense to prevent infection and eliminate infective pathogens in the body. The mammalian immune system can be divided in two branches: innate immunity, and acquired or adaptive immunity (Table 2. 1) (Akira et al., 2006). The innate immune response is the first line of host defense against pathogens and is mediated by phagocytes including macrophages and dendritic cells (DCs) (Kumar et al., 2011). Acquired or adaptive immunity is involved in the elimination of pathogens in the late phase of infection as well as the generation of immunological memory. The main cells involved in the acquired or adaptive immune response are T and B lymphocytes (Mackay et al., 2000).

Table 2. 1: Innate and adaptive immunity. Adapted from Janeway and Medzhitov (2002).

Property	Innate immune system	Adaptive immune system
Receptors	Fixed in genome	Encoded in gene segments
	Rearrangement is not necessary	Rearrangement is necessary
Distribution	Non-clonal	Clonal
	All cells of a class identical	All cells of a class distinct
Recognition	Conserved molecular patterns	Detailed molecular structures
Self-Nonself discrimination	Perfect: selected over evolutionary time	Imperfect: selected in individual somatic cells
Action time	Immediate activation of effectors	Delayed activation of effectors
Response	Co-stimulatory molecules	Clonal expansion or anergy
	Cytokines	Cytokines
	Chemokines	

The innate immunity is an evolutionary ancient part of the host defense mechanisms, the same molecular modules are found in plants and animals, meaning it arose before the split into these two kingdoms (Hoffmann et al., 1999). It is believed that the innate immune system predates the adaptive immune response on several grounds. First, innate host defenses are found in all multicellular organisms, whereas adaptive immunity is found only in vertebrates. Second, innate immune recognition distinguishes self from non-self perfectly. Third, the innate immune system uses receptors that are ancient in their lineage, whereas adaptive immunity appears to use the same effector mechanisms guided by clonally specific antibodies and T-cell receptors (TCR) encoded in rearranging genes of the Ig gene superfamily (Medzhitov and Janeway, 1997). The virtues of having an innate immune system of pathogen recognition lies not only in the delaying tactics of inflammation upon infection, but also in the activation of the adaptive immune system only when the body is under attack by a specific pathogen (Janeway and Medzhitov, 2002).

Innate immunity

Innate immunity covers many areas of the host defense against pathogenic microbes and viruses, including the recognition of pathogen-associated molecular patterns (PAMPs) (Janeway, 1989). In vertebrates, which are the only phylum that can mount an adaptive immune response, there are also mechanisms to inhibit the activation of innate immunity (Janeway and Medzhitov, 2002). Innate immunity lies behind most inflammatory responses, and are triggered in first instance by macrophages, polymorphonuclear leukocytes and mast cells through their innate immune receptors (Janeway and Medzhitov, 2002).

Invasion of a host by a pathogenic infectious agent triggers a battery of immune responses through interactions between a diverse array of pathogen-borne virulence factors and the immune surveillance mechanisms of the host. Host-pathogen interactions are generally initiated via host recognition of conserved molecular structures known as pathogen-associated molecular patterns (PAMPs) that are essential for the life cycle of the pathogen (Janeway and Medzhitov, 2002; Kumar et al., 2011). However, these PAMPs are either absent or compartmentalized inside the host cell, and are sensed by the host's germline encoded pattern recognition receptors (PRRs), which are expressed on innate immune cells such as dentritic cells, macrophages and neutrophils (Blasius and Beutler, 2010; Kawai and Akira, 2010; Medzhitov, 2007; Takeuchi and Akira, 2010). One advantage of these germ-line-encoded receptors is that they evolved by natural selection to have defined specificities for infectious microorganisms (Mackay et al., 2000). The strategy of the innate immune response may not be to recognize every possible antigen, but rather to focus on a few, highly conserved structures present in large groups of microorganisms (Janeway, 1989). Effective sensing of PAMPs rapidly induces host immune responses via the activation of complex signaling pathways that culminate in the induction of inflammatory responses mediated by various cytokines and chemokines, which subsequently facilitate the eradication of the pathogen (Kumar et al., 2009a).

The innate immune system uses a variety of pattern recognition receptors that can be expressed on the cell surface, in intracellular compartments, or secreted into the bloodstream and tissue fluids (Medzhitov and Janeway, 1997). The principle functions of PRRs include opsonization, activation of complement and of coagulation cascades, phagocytosis, activation of pro-inflammatory signaling pathways, and induction of apoptosis of the infected cells. Functionally, PRRs can be divided into three classes: secreted, endocytic and signaling.

Secreted PRR molecules function as opsonins by binding to microbial components and flagging them for recognition to the complement system and phagocytoses (Hawlisch and Köhl, 2006; Laarman et al., 2010; Morgan et al., 2005). The best characterized receptor of this class is the mannose-binding lectin (MBL), a member

of the calcium-dependent lectin family that binds to microbial carbohydrates to initiate the lectin pathway of complement activation (Fraser et al., 1998).

Endocytic pattern-recognition receptors occur on the surface of phagocytes. Upon recognition of a PAMP on a microbial cell, these receptors mediate the uptake and delivery of the pathogen into lysosomes. Pathogen-derived proteins can then be processed, and the resulting peptides can be presented by the major-histocompatibility-complex (MHC) molecules on the surface of the macrophages (Mackay et al., 2000). The macrophage mannose receptor, which is also a member of the calcium-dependent lectin family, is an endocytic PRR. It specifically recognizes carbohydrates with large numbers of mannoses, which are typical for bacteria (Suzuki et al., 1997; Thomas et al., 2000).

Signaling receptors recognize PAMPs and activate signaling-transduction pathways that induce the expression of a variety of immune-response genes, including inflammatory cytokines (Kawai and Akira, 2010; Kumar et al., 2009b; Ozinsky et al., 2000; Takeuchi and Akira, 2010). Toll-like receptors (TLRs) are the most widely studied PRRs and are considered to be the primary sensors of pathogens (Kumar et al., 2011).

Toll-like receptors

Toll-like receptors (TLRs) are evolutionarily conserved from the worm *Caenorhabditis elegans* to mammals (Akira and Takeda, 2004; Beutler, 2004; Hoffmann, 2003; Janeway and Medzhitov, 2002). Toll, the founding member of the TLR family, was initially identified as a gene product essential for the development of embryonic dorsoventral polarity in *Drosophila* (Belvin and Anderson, 1996; Hashimoto et al., 1988). It was later shown to play a critical role in the antifungal response of flies (Lemaitre et al., 1996). In humans, ten TLR family members have been identified. TLR1 to 9 are conserved in both humans and mice. TLR10 is expressed in humans but not in mice, whereas TLR11 is expressed in mice but not in humans (Kawai and Akira, 2010). TLRs are type I integral membrane glycoproteins characterized by the extracellular domains containing varying numbers of leucine-rich-repeat (LRR) motifs and a cytoplasmic signaling domain homologous to that of the interleukin-1 receptor (IL1R), termed the Toll/IL1R homology (TIR) domain (Bowie and O'Neill, 2000). Based on their primary sequence, TLRs can be further divided into several subfamilies, each of which recognizes related PAMPs: the subfamilies of TLR1, TLR2 and TLR6 recognize lipids, whereas the highly related TLR7, TLR8 and TLR9 recognize nucleic acids (Table 2. 2).

Table 2. 2: TLR recognition of bacterial or viral components. Adapted from Akira *et al*. (2006).

TLR	PAMPs	Species	Reference
Bacteria			
TLR4	LPS	Gram-negative bacteria	Poltorak et al. (1998) and Shimazu et al. (1999)
TLR6/TLR2	Diacyl lipopeptides	*Mycoplasma*	Thoma-Uszynski et al. (2001)
TLR1/TLR2	Triacyl lipopeptides	Bacteria and mycobacteria	Thoma-Uszynski et al. (2001)
TLR2	Lipoteichoic acid (LTA)	Group B Streptococcus	Alexopoulou et al. (2002); Ozinsky et al. (2000); Takeuchi et al. (2001, 2000)
TLR2	Peptidoglycan (PG)	Gram-positive bacteria	Alexopoulou et al. (2002); Ozinsky et al. (2000); Takeuchi et al. (2001, 2000)
TLR2	Porins	Neisseria	
TLR2	Lipoarabinomannan	Mycobacteria	Gilleron et al. (2003)
TLR5	Flagellin	Flagellated bacteria	Hayashi et al. (2001)
TLR9	CpG-DNA	Bacteria and mycobacteria	Hemmi et al. (2002)
Viruses			
TLR9	DNA	Viruses	Hochrein et al. (2004); Krug et al. (2004a, 2004b); Lund et al. (2003); Tabeta et al. (2004)
TLR3	dsRNA	Viruses	Alexopoulou et al. (2002)
TLR7 and TLR8	ssRNA	RNA viruses	Diebold et al. (2004); Heil et al. (2004); Hemmi et al. (2002)
TLR4	Envelope proteins	RSV, MMTV	Kurt-Jones et al. (2000)
TLR2	Hemagglutinin protein	Measles virus	Bieback et al. (2002); Compton et al. (2003)
TLR2	ND	HCMV, HSV1	Kurt-Jones et al. (2004)

Legend: RSV: respiratory syncytial virus; MMTV: mouse mammary tumor virus; HCMV: human cytomegalovirus; HSV1: herpes simplex virus 1; ND: not determined.

The first indication that mammalian TLRs may function as PRRs came with the description of a human homologue of *Drosophilla* Toll, now known as TLR4 (Medzhitov R et al., 1997). TLR4 recognizes LPS (Hoshino et al., 1999; Poltorak et al., 1998; Qureshi et al., 1999), but is not the sole receptor involved in the LPS recognition. Capture and transport of LPS molecules in the serum is mediated by LPS-binding protein (LBP)(Ulevitch and Tobias, 1995). At the plasma membrane, LBP is thought to transfer LPS monomers to CD14; a glycophosphatidylinositol (GPI) linked cell surface protein. It is not clear how CD14 facilitates recognition of LPS by TLR4, but its crucial role has been underscored by the LPS-hyporesponsive phenotype of CD14-deficient mice (Haziot et al., 1996; Moore et al., 2000). In order to complete the LPS recognition complex, MD-2 also plays an important role (da Silva Correia et al., 2001; Lien et al., 2000; Poltorak et al., 2000). This protein lacks a transmembrane anchor but is associated with the extracellular region of TLR4 (Shimazu et al., 1999).

TLR2 recognizes the largest number of ligands among the mammalian TLRs and perhaps of all PRRs (Janeway and Medzhitov, 2002). These include peptidoglycans (PG)(Schwandner et al., 1999; Takeuchi et al., 1999), bacterial lipoproteins (Aliprantis, 1999; Brightbill, 1999; Takeuchi et al., 2000b), a phenol soluble factor of *Staphylococcus epidermidis* (Hajjar et al., 2001), LPS from *Porphyromonas gingivalis* (Hirschfeld et al., 2001) and *Leptospira interrogans* (which differs in structure from the LPS of Gram-negative bacteria)(Werts et al., 2001), glycosylphosphatidylinositol lipid from *Trypanosoma cruzi* (Campos et al., 2001) and zymosan, a component of yeast cell walls (Underhill et al., 1999). TLR2 does not recognize these PAMPs independently, but functions by forming heterodimers with either TLR1 or TLR6 (Ozinsky et al., 2000; Takeuchi et al., 2001). This cooperation leads to the increased repertoire of ligand specificities.

TLR5 recognizes flagellin, the protein subunit that makes up bacterial flagella (Hayashi et al., 2001). Flagellin is a protein and does not contain any obvious features to flag it as nonself or pathogen-associated, unlike most other PAMPs (*e.g.* LPS or PG) it is very conserved at the N- and C-terminal ends that form its hydrophobic core, and therefore it is likely that this conserved region is recognized by TLR5 (Janeway and Medzhitov, 2002).

Unmethylated CpG DNA was long known for its immunostimulatory effects (Hartmann and Krieg, 1999; Heeg and Zimmermann, 2000; Lund et al., 2003; Tabeta et al., 2004). The logic of this recognition is that most of the mammalian genome is methylated at CpG sites, while bacteria lack CpG methylation enzymes (Krieg, 2000). Unlike most TLRs, which are expressed on the cell surface, TLR9 localizes intracellularly (Häcker et al., 1998; Krieg et al., 1995).

Toll signaling pathways

The engagement of TLRs by microbial components triggers the activation of signaling cascades, leading to the induction of genes involved in antimicrobial host defense, including inflammatory cytokines and chemokines, antimicrobial peptides, costimulatory molecules, MHC molecules, and other effectors necessary against the invading pathogen (Janeway and Medzhitov, 2002). After ligand binding, TLRs dimerize and undergo conformational changes required for the recruitment of TIR-domain-containing adapter molecules to the TIR domain of the TLR (Figure 2. 1). There are four adaptor molecules, namely MyD88, TIR-associated protein (TIRAP)/MyD88-adaptor like (MAL), TIR-domain-containing adaptor protein-inducing IFN-ß (TRIF)/TIR-domain-containing molecule 1 (TICAM1)(Oshiumi et al., 2003; Yamamoto et al., 2002), and TRIF-related adaptor molecule (TRAM)(Akira et al., 2006). The recruitment of these adaptor molecules activates various transcription factors such as NF-κB, IRF3/7, and MAP kinases to induce the production of pro-inflammatory cytokines and type I interferons (Kumar et al., 2011). The signaling pathways activated by TLR are similar to the ones activated by the IL1R and identical molecules comprise the two signaling cascades (Kopp and Medzhitov, 1999). All TLRs, except TLR3, recruit MyD88 and initiate MyD88-dependent signaling to activate NF-κB and MAP kinases to induce pro-inflammatory cytokines in macrophages and conventional DCs (cDCs). In addition to MyD88, TLR1, TLR2, TLR4 and TLR6 recruit TIRAP which serves as a linker adaptor between the TIR domains of the TLR and MyD88 (Kumar et al., 2009b, 2011).

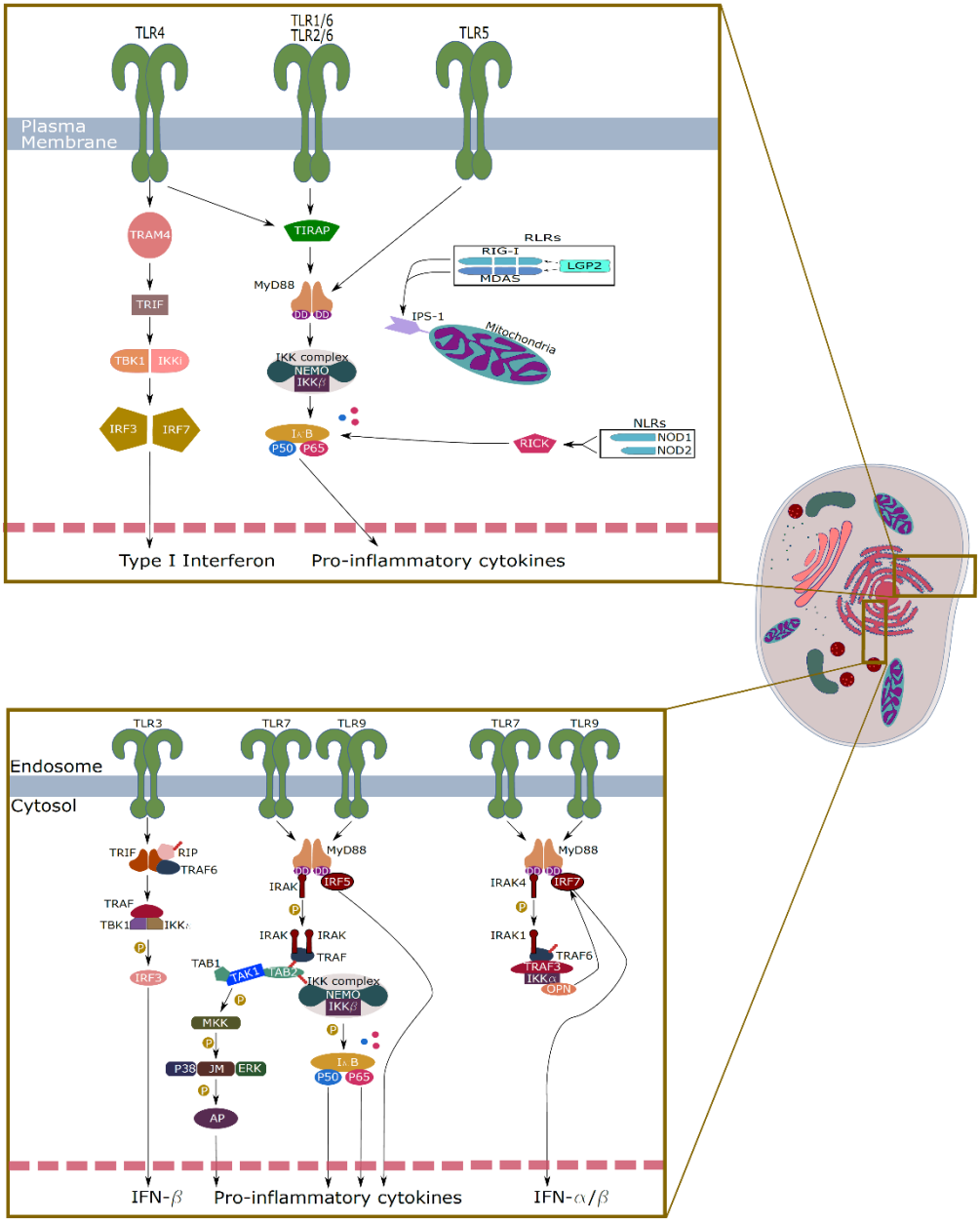
Upon ligation of TLR4, the adaptor MyD88 is recruited to the receptor complex (Medzhitov et al., 1998; Muzio et al., 1998). MyD88 has a C-terminal TIR domain that mediates its homophilic interaction with the receptor and an N-terminal death domain that engages the death domain of its downstream target IRAK (Wesche et al., 1997). Upon association with MyD88, IRAK, a serine threonine kinase, undergoes autophosphorylation. The RING-finger containing adaptor TRAF6 is also part of this activated signaling complex (Cao et al., 1996). It has been suggested that TRAF6 functions as an E3 ligase to ubiquitinate an as-yet-unidentified target that is necessary for TLR- and IL1R-mediated IκB kinase ß (IKK-ß) (Deng et al., 2000). Activated IKK phosphorylates and targets the NF-κB inhibitor IκB for degradation, thereby freeing NF-κB to translocate to the nucleus and turn on transcription of target genes (Ghosh et al., 1998).

RIG-I-like receptors

The RIG-I-like receptor (RLR) family consist of three members, namely RIG-I, MDA5 and LGP2. These sensors recognize the RNA from RNA viruses in the cytoplasm of infected cells and induce inflammatory cytokines and type I interferons (Figure 2. 1). This leads to the recruitment of macrophages and dendritic cells (Kumar et al., 2009a; Takeuchi and Akira, 2010; Wilkins and Gale, 2010).

Activation of RLR leads to the induction of type I interferons, which consist of several structurally related IFN-α proteins and a single IFN-ß protein, which can bind directly to infected cells in an autocrine or paracrine manner through a common receptor and initiate the transcription of several interferon-stimulated genes (ISGs). Type I interferons, together with ISGs, induce an antiviral state in all infected and healthy cells by altering various cellular processes. This inhibits viral replication, induces apoptosis in infected cells, increases the lytic capacity of natural killer cells, up-regulates the expression of MHC class I molecules and activates various components of the adaptive immune response (Kumar et al., 2011).

Figure 2. 1: Pattern recognition receptor (PRR) mediated signaling. ***Toll-like receptor (TLR) signaling*** | Recognition of PAMPs by plasma membrane-localized TLRs such as TLR2 (forms a heterodimer with TLR1 or TLR6 to from a functional receptor complex), TLR4, and TLR5 and endosomal-localized TLRs, such as TLR3, TLR7 and TLR9, activates TLR signaling pathways. All TLRs, except TLR3, recruit MyD88 and activate MyD88-dependent signaling. TLR1, 2, 4 and 6 recruit the additional adaptor molecule, TIRAP, for the recruitment of MyD88. TLR3 recruits TRIF and activates TRIF-dependent signaling. TLR4 also activates TRIF-dependent signaling through an additional adaptor molecule, TRAM. In cDCs, MyD88-dependent signaling is initiated through the recruitment and activation of various signaling molecules, such as IRAK family proteins, TRAF6 and TAK1 that activate the IKK complex. The IKK complex activates NF-κB subunits (*i.e.* P50 and P65) to initiate the transcription of inflammatory cytokine genes. In pDCs, the TLR7 and TLR9-mediated signaling pathways activate NF-κB via a MyD88-dependent signaling pathway in the same manner as for cDCs. cDCs stimulated with TLR3 PAMPs activate the TRIF-dependent signaling pathway through recruitment of TRIF to induce transcription of inflammatory cytokines and type I interferons through IKK complex and TBK1/IKKi, respectively, via the activation of NF-κB and IRF3/IRF7. ***RIG-I-like receptor (RLR) signaling*** | Recognition of PAMPs by cytosolic sensors, such as RIG-I and Mda5, activates signaling through the mitochondria-localized adaptor protein IPS-1 leading to the activation of NF-κB and IRF3/IRF7 through the IKK complex and TBK1/IKKi, respectively, which results in the production of inflammatory cytokines and type I interferon. LGP2, also a member of the RLR family, potentiates the RIG-I- and MDA5-mediated signaling pathway. ***NOD-like receptor (NLR) signaling*** | Recognition of PAMPs by NOD1 and NOD2 initiates the recruitment of RICK, which activates NF-κB via the IKK complex. Another member of the NLR family constitutes the inflammasome. The inflammasome is a multi-protein complex required for the maturation or activation of pro-IL1 family cytokines to its bioactive IL1 family cytokine. Figure adapted from Kumar *et al.* (2011, 2009a and 2009b) and Takeuchi and Akira (2010).


TLR4
TLR1/6
TLR2/6
TLR5
Plasma Membrane
TRAM4
TIRAP
RLRs
RIG-I
LGP2
MDA5
MyD88
TRIF
IPS-1
Mitochondria
TBK1
IKKi
IKK complex
NEMO
IKKβ
IRF3
IRF7
IκB
P50
P65
RICK
NLRs
NOD1
NOD2
Type I Interferon
Pro-inflammatory cytokines
TLR3
TLR7
TLR9
TLR7
TLR9
Endosome
Cytosol
TRIF
RIP
TRAF6
MyD88
IRAK
IRF5
TRAF
TBK1
IKKi
IRF3
IRAK
IRAK
TRAF
TAB1
TAK1
TAB2
IKK complex
NEMO
IKKβ
MKK
P38
JM
ERK
AP
IκB
P50
P65
MyD88
IRAK4
IRF7
IRAK1
TRAF6
TRAF3
IKKα
OPN
IFN-β
Pro-inflammatory cytokines
IFN-α/β

NOD-like receptors

NOD-like receptors (NLRs) are a family of PRRs that sense a wide range of ligands within the cytoplasm of cells and comprises 23 members in humans (Franchi et al., 2009; Kumar et al., 2009a; Shaw et al., 2008; Takeuchi and Akira, 2010). Proteins in this family possesses LRRs that mediate ligand sensing, a nucleotide binding oligomerization domain (NOD) and a domain for the initiation of signaling (*e.g.* CARDs, PYRIN, or baculovirus inhibitor of apoptosis repeat (BIR) domains (Inohara et al., 2005; Martinon and Tschopp, 2005). The most well studied NLRs are NOD1 and NOD2, which comprise of C-terminal LRRs, a central oligomerization domain and an N-terminal domain containing either one (NOD1) or two (NOD2) CARDs. These proteins are mainly expressed in the cytosol of various cells (Barnich et al., 2005; Kufer et al., 2008). NOD1 and NOD2 detect γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP) and muramyl dipeptide (MDP), found in bacterial PG (Chamaillard et al., 2003; Richardella et al., 2010). Ligand binding to NOD1 and NOD2 causes their oligomerization and results in NF-κB activation through the recruitment of RIP2/RICK, a serine/threonine kinase, to the NODs via their respective CARD domains by hydrophobic interactions (Figure 2. 1)(Akira et al., 2006).

Complement system

Complement is a major component of the innate immune system, and is involved in defending the host against pathogens through complement fragments that participate in opsonization, chemotaxis and activation of leukocytes (Rus et al., 2005). The complement proteins also play an important role in modulating adaptive immunity (Carroll, 2000). The complement system is a general term attributed to more than 30 soluble plasma and body fluid proteins and to a number of cell receptors and control proteins found in blood and tissues. The activation of the complement system provides a cascade-like defense barrier against bacteria, viruses, virus-infected cells, parasites, and tumor cells (Tegla et al., 2011). The complement system can be divided in three pathways, *i.e.* the classical, the alternative, and the lectin pathway (Figure 2. 2). All three pathways converge at the point of C3 cleavage and then generate the membrane attack complex C5b-9, leading to bacterial cytolysis (Rus et al., 2005).

Complement plays an important role in humoral immunity by enhancing both co-receptor signaling on B cells and antigen retention in follicular dendritic cells (Fearon, 2000). Particularly C3 plays multiple roles in regulating the B cell response to antigen (Morgan et al., 2005). Its activated form (C3b) binds covalently to the target (*e.g.* antigen), thus tagging it for recognition by the host. Bound C3b is proteolytically processed to smaller fragments, C3dg and C3d, which serve as ligands for CD21 on B cells and follicular dendritic cells (Cherukuri et al., 2004; Rus et al., 2005). Moreover, complement retention on the follicular DCs enhances the generation of antibody responses and the maintenance of immunologic memory (Youd et al., 2002).

The classical pathway is initiated by C1q binding, primarily to antigen-antibody complexes (IgG and IgM) but also to viral envelopes, Gram-negative bacterial walls and C-reactive protein (CRP). Activation of C1r and C1s, with generation of C1s esterase, is followed by cleavage of C4 and C2. This cleavage releases small anaphylactic peptides (C4a and C2b) and allows the assembly of the C3 convertase, C4bC2a. The C3 convertase then cleaves C3, generating C3b and C3a, and C3b binding to C4b generates the C5 convertase, C4b2a3b. C3b and its further cleavage products, iC3b and C3dg, can interact with complement receptors type 1 (CR1), 2 (CR2) and 3 (CR3) (Ahearn and Fearon, 1989; Krych-Goldberg and Atkinson, 2001).

In the alternative pathway, C3 is spontaneously activated at low levels, and the resulting C3B covalently attaches to both host cells and pathogens. Host cells produce a series of proteins, such as factors H and I, that cause C3b cleavage and Bb decay (Xu et al., 2001), as such preventing the complement reaction from proceeding on their cell surface. Because pathogens lack these proteins, they are singled out for destruction (Alberts et al., 2002). The activation of a serine protease, factor D, cleaves factor B into Ba and Bb, when factor B is complexed with spontaneously hydrolyzed C3b. Bb is a serine protease that generates the C3 convertase (C3bBb) of the alternative pathway, whose role is to cleave C5 and of which the activity is increased by properdin.

The lectin pathway is initiated by binding of mannose-binding lectin (MBL) and ficolins to carbohydrate groups on the surface of bacterial cells (Fujita et al., 2004). Mannose-binding lectin is a serum protein that forms clusters of six carbohydrate-binding heads around a central collagen-like stalk. This assembly binds specifically to mannose and fucose residues in bacterial cell walls that have the correct spacing and orientation to match up perfectly with the six carbohydrate-binding sites (Alberts et al., 2002).

Mannose-binding lectin and ficolins are the pattern-recognition molecules typical of the lectin pathway and serve to attach the MBL-associated serine proteases (MASP) 1, 2 and 3, thus activating MASP esterase activity. Upon activation, MASPs cleave and activate C4 and C2, thus generating the C3 convertase, C4bC2a (Dahl et al., 2001; Petersen et al., 2000). Activation of the classical or lectin pathways also activates the alternative pathway through a positive feedback loop, amplifying their effects.

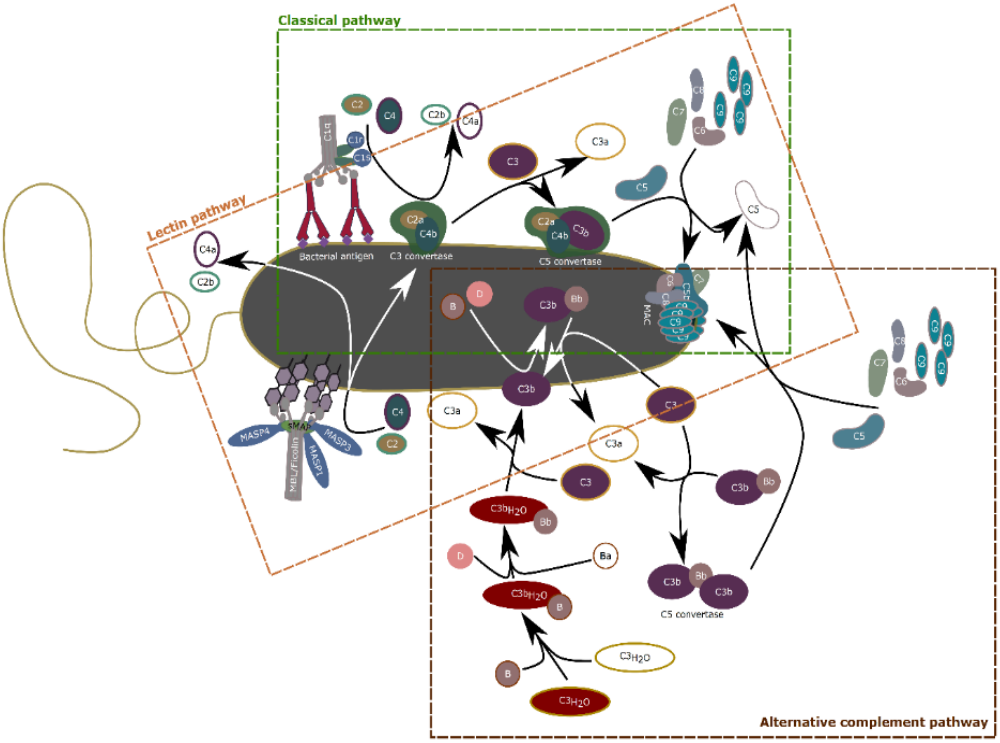


Figure 2. 2: Activation pathways of the complement system. ***The classical pathway*** is initiated by the binding of the C1 complex to antibody already bound to antigen, leading to the formation of C4b2a enzyme complex (the C3 convertase). The C1 complex consists of C1q and two molecules, each of C1r and C1s. The binding of the recognition subcomponent C1q to the Fc portion of immunoglonulins results in the autoactivation of the serine protease C1r. C1r then cleaves and activates C1s, which translates the activation of the C1 complex into complement activation through the cleavage of C4 and C2 to form a C4bC2a enzyme complex. C4bC2a acts as a C3 convertase and cleaves C3. ***The lectin pathway*** is activated by binding of either mannose binding lectine (MBL) or Ficolin and MAPS1, 2, and 3 to an array of carbohydrate groups (mannose) groups on the surface of the bacterial cells. MASP2, similar to C1s, is responsible for the activation of C4 and C2, which leads to the generation of the same C3 convertase (C4bC2a) as in the classical pathway. MASP1 is able to cleave C3 directly. ***The alternative pathway*** is initiated by the low-grade activation of hydrolyzed C3 ($C3_{H_2O}$) and activated factor B (Bb). The activated C3b binds factor B (B), which is then cleaved into Bb by factor D (D) to form the alternative pathway C3 convertase, C3bBb. Once C3b is attached to the cell surface, the amplification loop consisting of the alternative-pathway components is activated, and the C3-convertase enzymes cleaves many molecules of C3 to C3b, which bind covalently around the site of complement activation. Generation of C3 convertase allows the formation of the C5 convertase, which initiates the formation of the C5b9 terminal complement complex, *i.e.* the membrane attack complex (MAC). Figure adapted from Fujita (2002), Rus *et al*. (2005) and Tegla *et al*. (2011).

Membrane-immobilized C3b molecules, produced by any of the three pathways, trigger a further cascade of reactions that leads to the assembly of the late components of the complement reaction to form membrane attack complexes. The activation of C5 through C9 and the assembly of C5b-9 begins when the C5 convertase (*i.e.* C4b2a3b and C3bBb) cleaves C5 to generate C5a and C5b. C5b then undergoes a conformational change, exposing a metastable binding site for C6. The C5b6 complex can then bind reversibly to the cell membrane. Subsequently, the interaction of C7, C8 and C9 with C5b6 complexes leads to the assembly of a supramolecular C5b-9 complex, which is able to form transmembrane pores (Müller-Eberhard, 1988). This C5b-9 complex, which is effective in inducing cell lysis, is also called the membrane attack complex (MAC) (Tegla et al., 2011).

Adaptive immunity

Adaptive immunity is a relative newcomer on the evolutionary landscape. Because the mechanism of generating receptors in the adaptive immune system involves great variability and rearrangement of receptor gene segments, the adaptive immune system can provide specific recognition of foreign antigens, immunological memory of infection, and pathogen-specific adaptor proteins. However, the adaptive immune response is also responsible for allergy, autoimmunity and the rejection of tissue grafts (Janeway and Medzhitov, 2002). The adaptive immunity adds specific recognition of proteins, carbohydrates, lipids, nucleic acids and pathogens to the underlying innate immune system. The two systems, *i.e.* innate and adaptive immune system, are linked through the use of the same effector cells (dendritic cells or macrophages) (Janeway, 1989).

Activation of the adaptive immune system occurs upon pathogen recognition by antigen presenting cells (APCs), that play a pivotal role at the interface of innate and adaptive immunity (Pulendran et al., 2001). Immature dendritic cells reside in the peripheral tissues, where they actively sample their environment by endocytosis and micropinocytosis. Upon encountering a pathogen, they undergo a developmental program called dendritic cell maturation, which includes induction of costimulatory activity, antigen processing, increased MHC molecule expression, and migration to the lymph nodes, where they can prime naïve antigen-specific T cells (Orsini et al., 2003).

Unlike the innate mechanisms of host defense, the adaptive immune system manifests exquisite specificity for its target antigens. Adaptive responses are based primarily on the antigen-specific receptors expressed on the surface of T and B lymphocytes. The antigen-specific receptors of the adaptive response are encoded by genes that are assembled by somatic rearrangement of germline gene elements to form intact T-cell receptor (TCR) and immunoglobulin (B-cell antigen receptor) genes. This is in contrast to the germline-encoded recognition molecules of the innate immune response. The assembly of antigen receptors from a collection of a few hundred germline-encoded gene elements permits the formation of millions of

different antigen receptors, each with potentially unique specificity for a different antigen (Bonilla and Oettgen, 2010; Chaplin, 2010; Schroeder and Cavacini, 2010). The adaptive immunity is mediated by immunoglobulins and T cell receptors (TCRs), which are generated through the recombination of variable (V), diversity (D) and joining (J) gene segments (Tonegawa, 1983). The V(D)J recombination process depends on the recognition of recombination signal sequences (RSSs), which flank the segmental elements and this creates extensive variation in the receptor structure at junctional (joining) interfaces. The V(D)J rearrangement form of somatic recombination occurs in the progenitors of B and T cells and is mediated by recombination-activating genes 1 (RAG1) and 2 (RAG2), which function in a lymphocyte- and site-specific recombinase complex and are supported by ubiquitous DNA repair factors (Gellert, 2002).

A major challenge faced by the immune system is to identify host cells that have been infected by microbes that subsequently use the cell to multiply within the host. A major role of the T-cell arm of the immune response is to identify and destroy infected cells. T cells can also recognize peptide fragments of antigens that have been taken up by antigen presenting cells (APCs) through the process of phagocytosis or pinocytosis. The immune system permits T cells to recognize infected host cells by the recognition of both a self-component and a microbial structure. This is mediated through the use of major histocompatibility (MHC) molecules. MHC molecules (also called HLA antigens) are cell-surface glycoproteins that bind peptide fragments of proteins that either have been synthesized within the cell (class I MHC molecules) or that have been ingested by the cell and proteolytically processed (class II MHC molecules) (Chaplin, 2010; Davis and Bjorkman, 1988; Menéndez-Benito and Neefjes, 2007; Watts, 2004).

Antigen presentation: Major histocompatibility molecules

Class I MHC molecules

There are three major MHC class I molecules, *i.e.* HLA-A, HLA-B and HLA-C. The class I HLA molecules are cell-surface heterodimers consisting of a polymorphic transmembrane α chain associated with the nonpolymorphic $ß_2$-microglobulin protein (Bjorkman, 1997). It is the α chain that determines whether the class I molecule is an HLA-A, HLA-B or HLA-C molecule. The α-chain gene encodes three extracellular domains (α_1, α_2 and α_3), a transmembrane domain that anchors the protein in the cell membrane and a short intracellular domain. The α_1 and α_2 domains associate with each other, forming a groove in which the antigenic peptide can bind (Cresswell et al., 1999; Monaco, 1992; Rammensee et al., 1993).

The antigenic peptides that are found bound in the peptide-binding groove of the MHC class I molecules are derived from proteins synthesized within the cell (Figure 2. 3)(Huston, 1997).

The peptide fragments are generated from cellular proteins through the action of the proteasome (Niedermann, 2002). After exiting the proteasome, peptide fragments are transported into the endoplasmatic reticulum (ER) by the action of a specific multi-subunit transmembrane transporter, the transporter associated with antigen presentation (TAP). Once in the ER, the peptides are loaded into the class I protein-binding groove under the direction of the ER protein tapasin with the help of the calcium-binding chaperone protein calreticulin and the oxidoreductase Erp57 (Garbi et al., 2006; Momburg and Tan, 2002). Before its interactions with β_2-microglobulin, the class I protein is maintained in a conformation that favors the interaction with peptide fragments by association with chaperone protein calnexin. Interaction with β_2-microglobulin stabilizes the complex, causing dissociation of calnexin and permitting transport of the peptide-loaded class I molecule through the Golgi complex into exocytic vesicles that release the intact complexes onto the cell surface. This pathway is well adapted to delivering viral peptides produced in a virus-infected cell to the cell surface bound class I HLA molecules in a form that can be recognized by cytotoxic $CD8^+$ T cells (Melief, 2003; Sigal et al., 1999).

The complex forming the class I MHC molecule and antigenic peptide produces a composite structure that is the molecular target of the TCR. The TCR contacts both the antigenic peptide and the flanking α-helices of the peptide-binding groove. The TCR has no measurable affinity for the antigenic peptide alone and very low affinity for MHC molecules (Zinkernagel and Doherty, 1997). A key biological consequence of requiring the T cell to recognize antigenic peptides only when they are bound into the groove of an MHC molecule is that this permits the T cell to ignore free extracellular antigen and to focus rather on cells that contain the antigen. In the case of cells that are infected by a pathogenic microbe, this permits the T cells to focus their response on the infected cells. The α_3 domain of the class I heavy chain interacts with the CD8 molecule on cytolytic T cells. The binding of CD8 expressed by the T cell to the α_3 domain of the class I molecule expressed by the APC strengthens the interaction of the T cell with the APC and helps ensure that full activation of the T cell occurs (Joshi and Kaech, 2008).

Class II MHC molecules

Like the class I molecules, the class II MHC molecules consist of two polypeptide chains, but in this case both are MHC-encoded transmembrane proteins and are designated α and ß. There are three major class II proteins, *i.e.* HLA-DR, HLA-DQ and HLA-DP. Each chain of the class contains a short cytoplasmatic anchor, a transmembrane domain, and two extracellular domains designated for the α chain, α_1 and α_2, and for the ß chain,β_1 and β_2 (Bjorkman, 1997). When the α and ß chains pair, the α_1 and β_1 domains combine to form a peptide-binding groove very similar in structure to that formed by the association of the α_1 and α_2 domains of the MHC class I proteins. The α_2 and β_2 domains of the proteins provide support for this peptide-binding domain. The β_2 domain also interacts with CD4 expressed on the T_H cells, and enhances the interaction between these T cells and the class II-

expressing APCs in a fashion similar to the way that CD8 is bound to the MHC class I molecule and enhances the cytotoxic T-cell activation (König et al., 1996).

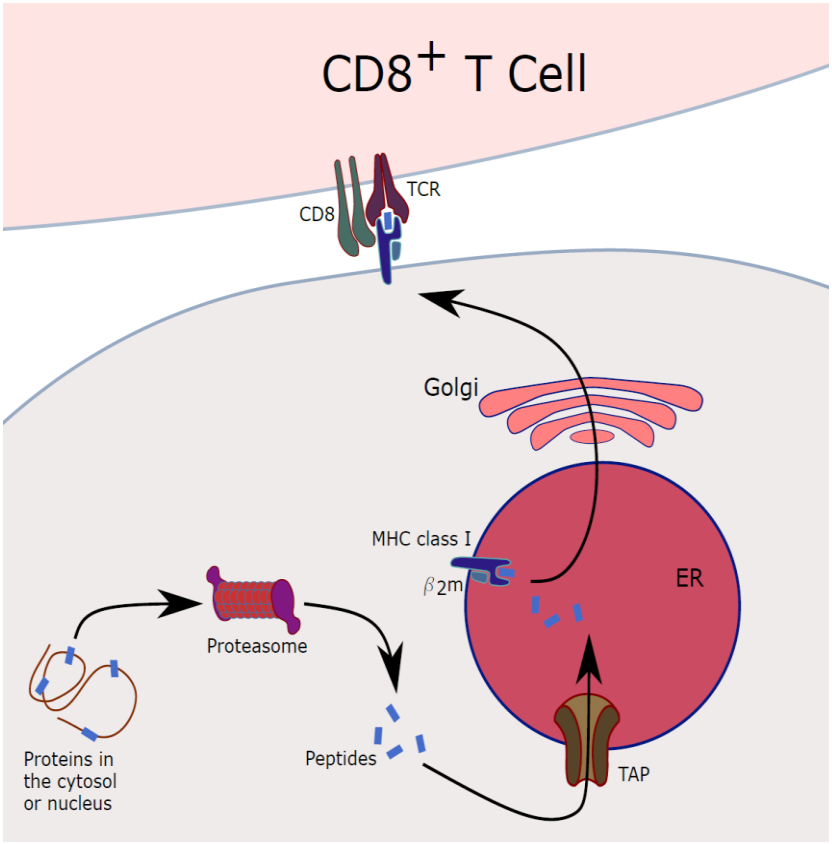


Figure 2. 3: The basic MHC class I antigen presentation pathway. The presentation of intracellular antigenic peptides by MHC class I molecules is the result of a series of reactions. First, antigens are degraded by the proteasome. Then, the resulting peptides are translocated via transporter associated with antigen presentation (TAP) into the endoplasmic reticulum (ER) lumen and loaded onto the MHC class I molecules. Peptides-MHC class I complexes are released from the ER and transported via the Golgi to the plasma membrane for antigen presentation to $CD8^+$ T cells. $ß_2m$, $ß_2$-microglobulin; TCR, T cell receptor. Figure adapted from Chaplin (2010) and Neefjes *et al*. (2011).

The class II proteins are expressed constitutively on the B-cells, dendritic cells, monocytes and macrophages. All these cells present antigens to $CD4^+$ T cells. Expression of MHC class II proteins can also be induced on many additional cell types, including epithelial and endothelial cells after stimulation with IFN-γ, permitting these cells to present antigens to $CD4^+$ T cells at sites of inflammation

(Dengjel et al., 2005; Dongre et al., 2001; Menéndez-Benito and Neefjes, 2007). Antigens that are presented by class II proteins are loaded into the class II peptide-binding groove through the "exogenous" pathway that starts by endocytosis or phagocytosis of extracellular proteins (Huston, 1997). The exogenous antigens include antigenic proteins of extracellular pathogens, such as most bacteria, parasites and virus particles that have been released from infected cells and taken up by phagocytosis (Figure 2. 4).

The ingested antigens are processed to linear peptide fragments by means of proteolysis after fusion of lysosomes with the phagocytic vacuoles or endosomes to form an acidic compartment (Turley, 2000). The peptide fragments then accumulate in the MHC II loading compartment, where they encounter nascent class II proteins. The α and ß chains of the class II molecules are synthesized in the ER. To protect the class II molecule's peptide-binding groove so that it can later accommodate an antigenic peptide, the α and ß chains associate with the nonpolymorphic invariant chain (Ii), assisted by the chaperone protein calnexin. A portion of the Ii chain designated class II-associated invariant chain peptide lies in the peptide-binding groove of the class II heterodimer, preventing binding of antigenic peptides. Once the class II-Ii complex has formed, it dissociates from calnexin and is transported to the class II loading compartment (Van Kaer, 2001). In the class II loading compartment, the bulk of the Ii is degraded by acid proteases, such as cathepsins, and exchange of the class II-associated invariant-chain peptide for an antigenic peptide is catalyzed by the action of the HLA-DM molecule, resulting in the formation of a mature class II protein (Sadegh-Nasseri et al., 2008; Van Lith et al., 2010). The class II proteins loaded with antigenic peptide are then delivered to the cell surface by means of fusion of the class II^+ endosome to the plasma membrane (Chaplin, 2010).

T and B cell receptors

V(D)J Recombination

V(D)J recombination, *i.e.* the recombination of variable (V), diversity (D) and joining (J) gene segments, is the specialized DNA rearrangement used by cells of the immune system to assemble T-cell receptor and immunoglobulin genes from preexisting gene segments (Figure 2. 5). Because there is a large choice of segments to join, this process accounts for much of the diversity of the immune response. V(D)J recombination is initiated by breakage at precisely defined locations in the DNA, but is then completed by a repair process related to the repair of DNA breaks caused by ionizing radiation or other genotoxic agents (Fugmann et al., 2000; Gellert, 1997, 2002; Lewis, 1994a; Sleckman et al., 1996).

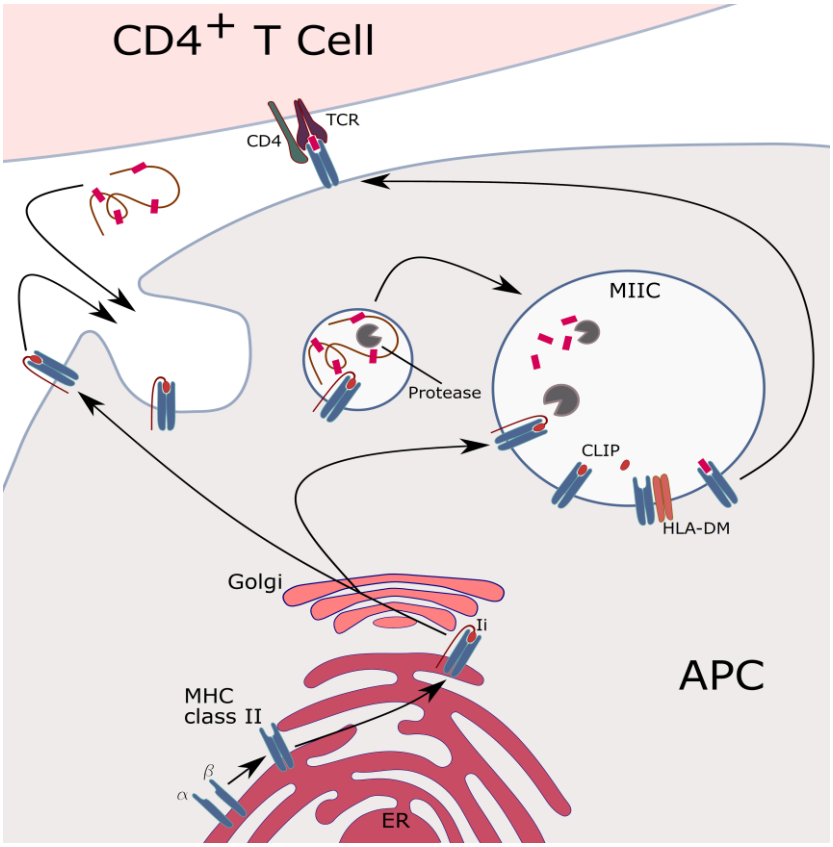


Figure 2. 4: The basic MHC class II antigen presentation pathway. MHC class II α- and ß-chains assemble in the endoplasmic reticulum (ER) and form a complex with the invariant chain (Ii). The Ii-MHC class II heterotrimer is transported through the Golgi to the MHC class II compartment (MIIC), either directly and/or via the plasma membrane. Endocytosed proteins (or pathogens) and Ii are degraded by resident proteases in the MIIC. The class II associated Ii peptide (CLIP) fragment of Ii remains in the peptide groove of the MHC class II dimer and is exchanged for an antigenic peptide with the help of the dedicated chaperone HLA-DM. MHC class II molecules are then transported to the plasma membrane to present antigenic peptides to $CD4^+$ T cells. APC, antigen-presenting cells; TCR, T cell receptor. Figure adapted from Chaplin (2010) and Neefjes *et al*. (2011).

Two types of nucleotide insertions are found in coding joints, nontemplated and templated. Nontemplated tracts up to 15 nucleotides in length (so-called N regions) are added by the enzyme called terminal deoxynucleotidyl transferase (TdT). Expression of TdT is normally limited to early lymphoid cells where V(D)J recombination is active and therefore these insertions are relatively specific to this type of recombination. TdT adds deoxynucleotides without a template to the ends of DNA chains, but with a preference for G residues that results in N regions being

generally GC-rich (Gilfillan et al., 1993; Komori et al., 1993). A templated type of nucleotide addition is also found in coding joints. These additions are more significant for the basic recombination mechanism. These palindromic nucleotide insertions add a few nucleotides complementary to the last bases of the coding end next to the RSS. They result from off-center nicking of the hairpin DNA intermediates that are formed at coding ends by the action of the RAG proteins (Lafaille et al., 1989; Lewis, 1994b).

The V(D)J recombination has two distinct stages. In the first stage, the RAG1 and RAG2 proteins cooperate to recognize the RSSs and to ensure their correct 12/23 pairing, and to break the DNA between each heptamer and the neighboring coding sequence. In the later stage, factors, that are also used in other types of nonhomologous end joining, process and link the ends into coding joints and signal joints (Gellert, 2002).

The later steps of V(D)J recombination have many aspects in common with general DNA double-strand break repair, and the two processes share a number of factors (*e.g.* DNA-dependent protein kinase (DNA-PK) Ku protein, DNA ligase IV, Xrcc4, Artemis, histone H2AX and the Mre11/Rad50/Nbs1 complex) (Jeggo, 1998; Steen et al., 1996). V(D)J recombination is regulated in at least two ways, first by the expression pattern of RAG1 and RAG2, and secondly by the limited access of the recombination machinery to particular DNA sites. RAG1 and RAG2 are normally expressed together, and only in early lymphoid cells (Nagaoka et al., 2000).

Class switching recombination

Antibody class, or isotype, is determined by the heavy chain constant (C_H) region, which is important for determining the antibody's effector function. The C_H region is bound by cell-surface receptors, *e.g.*, Fc receptors on many cell types, poly Ig receptors on mucosal epithelial cells, and by complement. Different C_H regions have different affinities for these proteins, thus greatly influence antibody function (Horikawa et al., 2007; Martin and Goodnow, 2002; Waisman et al., 2007).

Isotype switching occurs by an intrachromosomal deletional recombination event (Figure 2. 5). Class switch recombination (CSR) occurs between switch (S) regions located upstream of each of the C_H regions except Cδ and results in a change from IgM and IgD expression by naïve B cells to expression of one of the downstream isotypes. IgD expression occurs by alternative transcription termination/splicing of the Cμ – Cδ genes. S regions consist of tandem repeats of short G-rich sequences (20 – 80 bp), which differ for each isotype, with an overall length varying from ~1 kb to 12 kb, and CSR can occur anywhere within or near the S regions (Dunnick et al., 1993; Min et al., 2005). Class switch recombination occurs by end-joining type of recombination, rather than by homologous recombination (Manis et al., 2002; Stavnezer, 1996a).

Figure 2. 5: Antigen receptor diversification. **A|** Schematic of the B-cell receptor (BCR) heavy locus. Antigen receptor repertoire diversity is primarily established during lymphocyte development, during which V (green), D (brown), and J (blue) gene segments are rearranged through the process of V(D)J recombination. (Murphy et al., 2008). During the recombination process, nucleotides may be added or deleted at segment junctions (not shown), contributing to additional sequence diversity. In class-switch recombination, gene segments encoding constant regions (dark green) are rearranged resulting in the production of antibodies with different isotypes and corresponding effector functions. BCRs and TCRs are similarly organized. The TCRß locus undergoes similar V(D)J recombination as the BCR, with the exception of somatic hypermutation and class-switch recombination. **B|** BCRs are composed of two distinct subunit chains: light chain and heavy chain. The antigen binding surface is formed by the variable region of each chain, which is encoded by recombined V, J, and D heavy gene segments. Abbreviations: BCR, B cell receptor; TCR, T cell receptor; V, J, and D, Variable, Joining, and Diversity gene segments. Adapted from Calis and Rosenberg (2014).

B cells undergo antibody, or Ig, class switching *in vivo* after B-cell maturation which is driven by immunization or infection, or upon appropriate activation in culture. Engagement of the CD40 receptor on B cells by CD40L (CD154) provides crucial signaling for CSR (Stavnezer et al., 2008). The process of class-switching is partly under cytokine control, *e.g.* IL4 and IL13 promote switching to IgE and IFN-γ can antagonize this effect (Oettgen, 2000). IL10 and TGFß promote switching to IgA (Johansen and Brandtzaeg, 2004). Class switch recombination requires a minimum of two complete rounds of cell division for IgG and IgA CSR and perhaps additional rounds for IgE CSR (Deenick et al., 1999; Hasbold et al., 1998; Hodgkin et al., 1996; Rush et al., 2005). This requirement seems to be at least partially due to the requirements for induction of activation-induced cytidine deaminase (AID) expression (Rush et al., 2005). Naïve B cells have the potential to switch to any isotype, and cytokines secreted by T cells and other cells direct the isotype switching (Stavnezer, 1996a, 1996b).

Class switch recombination and somatic hyper mutation (SHM) are initiated by AID, which converts cytosines in S regions and Ig variable regions to uracils (dU) by deamination (Chaudhuri et al., 2003; Dickerson et al., 2003; Muramatsu et al., 2000; Petersen-Mahrt et al., 2002; Pham et al., 2003; Revy et al., 2000). Subsequent repair of the dU residues leads to single-stranded DNA breaks (SSBs) within the donor Sμ region and within an acceptor Sx region, to initiate the process of intrachromosomal DNA recombination. Removal of the dU residues by enzymes within the base excision repair (BER) pathway is required to introduce the DNA breaks necessary for CSR (Petersen-Mahrt et al., 2002; Rada et al., 2002; Schrader et al., 2005). BER consists of highly active ubiquitous DNA repair pathways for repairing oxidized and deaminated bases, which are generated more than 10^4 times per cell per day by oxidation, especially during inflammation, and by spontaneous hydrolysis (Christmann et al., 2003). There are four mammalian uracil DNA glycosylases in the BER pathway, UNG, SMUG1, MBD4 and TDG. It is UNG that excises the dU residue created by AID activity (Bardwell et al., 2003; Rada et al., 2002).

The BER enzyme that repairs the abasic sites left by UNG activity is apurinic/apyrimidinic endonuclease (APE), which incises the phosphate backbone of DNA at abasic sites, producing SSBs (Christmann et al., 2003). In the canonical BER pathway, the single-nucleotide gap generated by the action of UNG and APE is filled in by the DNA polymerase ß (Pol ß) (Barnes and Lindahl, 2004; Beard and Wilson, 2006). A second repair pathway, mismatch repair (MMR), contributes to CSR but is not essential. The major role of MMR in all cells is to correct misincorporated nucleotides during DNA synthesis (Kunkel and Erie, 2005). The most attractive model for the role of MMR during CSR is to convert SSBs that are not near each other on the opposite DNA strands to DSBs (Stavnezer and Schrader, 2006). If the SSBs that are introduced by AID-UNG-APE are near each other on opposite DNA strands, they can spontaneously form a DSB, but if not, the SSBs do

not destabilize the duplex and are simply repaired. As S regions are large and the breaks appear to occur anywhere within S regions (Dunnick et al., 1993; Min et al., 2005; Schrader et al., 2005), it seems unlikely that the SSBs would be sufficiently proximal to form a DSB most of the time. Mismatch repair could convert these distal SSBs to DSBs that are required for CSR. After DSB formation, 5' or 3' single-stranded overhangs remain. These tails must be excised or filled in to create blunt, or nearly blunt, DSBs appropriate for an end-joining recombination with the other S regions (Schrader et al., 2004). MMR normally recruits the processivity factor proliferating cell nuclear antigen (PCNA) and replicative DNA polymerase for fill-in synthesis of 5' overhangs (Kunkel and Erie, 2005). However, replicative Pol cannot replicate past an abasic site, resulting in the recruitment of error-prone translation Pols. After formation of the DSBs in the donor and acceptor S regions, the S regions are recombined using ubiquitous proteins that perform nonhomologous end-joining (NHEJ) in all cell types (Stavnezer et al., 2008).

T-cell immunity

The major class of T cells are defined by its surface expression of the αß T cell receptor (TCR). This receptor has evolved primarily to recognize peptide antigens presented in complex with class I or class II MHC proteins. αß T cells differentiate into several different subsets, $CD8^+$ T cells act primarily to kill cells infected with intracellular microbes, whereas $CD4^+$ T cells regulate the cellular and humoral immune response (Bonilla and Oettgen, 2010; Chaplin, 2010).

T-cell receptor

Each individual T cell bears antigen receptors of a single specificity. A repertoire of T cells that can protect against the vast universe of microbial pathogens must therefore include a very large number of cells encoding a huge array of discrete TCRs. These receptors are somatically assembled using VDJ recombination (Nguyen et al., 2007).

Selection of cells carrying functional TCR genes occurs in the thymus (Hedrick, 2008; Huston, 1997; Jenkinson et al., 2006; Miller, 2002; Takahama, 2006). The thymus contains three compartments, *i.e.* the subcapsular zone, the thymic cortex and the thymic medulla. The subcapsular zone is where bone marrow-derived prothymocytes begin to differentiate, proliferate, and rearrange their TCR ß chains. The cells then move to the thymic cortex, where the α chain gene elements rearrange, potentially forming a functional, mature αß TCR. In the cortex, the TCR are tested for a sufficient affinity for MHC molecules to permit them to ultimately recognize antigen-MHC complexes. This involves the interactions between the developing lymphocyte and the specialized cortical epithelium (Nitta et al., 2008). If the lymphocyte fails this positive selection, it undergoes apoptosis and is cleared by the thymic cortical macrophages. In the thymic medulla the thymocytes are screened for potential autoreactivity. This screening includes testing for reactivity for an extensive array of tissue-specific proteins that are expressed by a population

of thymic medullary epithelial cells under the control of a gene called autoimmune regulator (AIRE) (Mathis and Benoist, 2009). Cells that recognize self-peptides expressed by these epithelial cells are removed by means of apoptosis, and cells that have survived this negative selection are exported to the circulation.

During their progress through the thymus, αß T cells differentiate into discrete subpopulations, each with defined repertoires of effector functions. The major subsets are defined by their selective surface expression of CD4 and CD8. In the thymus, most developing T cells follow a developmental program in which they first express neither CD4 nor CD8 (double negative) and then express both CD4 and CD8 (double positive), in the cortex (von Boehmer et al., 1989). Double-positive cells are tested by means of positive selection in the thymic cortex, and those that are selected on class I MHC molecules become $CD4^{-}CD8^{+}$ and those selected on class II MHC molecules become $CD4^{+}CD8^{-}$. $CD4^{+}$ T cells are generally designated helper cells and activate both humoral immune responses (B-cell help) and cellular responses (delayed-type hypersensitivity responses). $CD8^{+}$ cells show a major cytotoxic activity against cells infected with intracellular microbes and against tumor cells but also contain regulatory cells that down-regulates immune responses (suppressor cells) (Chaplin, 2010).

Less than 5 % of the developing T cells survive positive and negative selection. Approximately 90 to 95 % of the circulating T cells use the αß TCR, the other 5 to 10 % use an alternate heterodimeric TCR composed of γ and δ chains. The γ and δ chains also assemble by means of RAG1/RAG2-mediated rearrangement of V, D (for the δ chain only) and J elements. A portion of the γδ T cells are generated in the thymus, but a major fraction appears to be generated in an extra thymic compartment, resulting in cells that largely populate the gastrointestinal tract (Ishikawa et al., 2007).

The antigen-specific α and ß chains of the TCR associate with invariant accessory chains that serve to transduce signals when the TCR binds to antigen-MHC complexes (Salmond et al., 2009). These accessory chains make up the CD3 complex, consisting of the transmembrane CD3γ, CD3δ and CD3ε chains plus a largely intracytoplasmic homodimer of two CD3ξ chains. Interaction of the TCR/CD3 complex with antigenic peptide presented in an HLA molecule provides only a partial signal for cell activation.

Although the basic principles of thymic development and the mechanisms of activation are shared by all T cells, there is a remarkable diversity of effector functions that are elicited in response to activation. T cells can play direct roles in elimination of pathogens by killing infected target cells. Moreover, they can also function as helper cells, providing cognate (involving direct cellular contact) or cytokine signals to enhance both B- and T-cell responses, as well as causing activation of mononuclear phagocytes. Finally, T-cells regulate the immune responses, limiting tissue damage incurred by means of autoreactive or overly

inflammatory immune responses (Bonilla and Oettgen, 2010). Both $CD4^+$ and $CD8^+$ T cells differentiate into functionally distinct subsets after exposure to antigen.

T-cell activation

Mature T-cells are activated on interaction of their TCRs with antigenic peptides complexed with MHC molecules. $CD8^+$ T cells can interact with peptides on almost any cell expressing MHC class I (Melief, 2003; Sigal et al., 1999), whereas the TCRs of $CD4^+$ T cells engage peptides bearing MHC class II (König et al., 1996). The T-cell activation is initiated when the TCR and associated proteins recognize a peptide/MHC complex on an APC, leading to rapid clustering of TCR-associated molecules at the physical interface between T cells and APCs and the formation of a so-called immunologic synapse (Dustin, 2009). The T-cell side of the synapse is focused around a central cluster of CD3 (γ, δ, ε and ξ) and TCR (α and ß), which bind specifically to the peptide/MHC complex, as well as CD4/CD8 molecules, which stabilize this interaction by binding to nonpolymorphic regions of MHC class I or MHC class II, respectively. Adhesion molecules known as integrins stabilize the synapse. The aggregation of these molecules in the synapse facilitates the early events in TCR signaling (Bonilla and Oettgen, 2010; Nurieva et al., 2009).

Simultaneous binding to MHC/peptide on the APCs by TCRs and CD4/CD8 in the synapse brings the cytosolic domains of these molecules into proximity. As a result, the CD4- and CD8-associated Src family protein tyrosine kinase Lck is able to phosphorylate tyrosine residues contained in cytoplasmic immunoreceptor tyrosine-based activation motifs of the TCR-associated CD3 chains. This results in the recruitment of the critical adaptor molecule, ξ-associated protein, 70 kd (ZAP-70), which binds to immunoreceptor tyrosine-based activation motif phosphotyrosines and phosphorylates a number of cytosolic proteins. This subsequently triggers the assembly of an intracellular complex of scaffolding and activated signaling proteins, including linker of activated T cells (LAT) and SH2-containing leukocyte protein, 76 kd (Bonilla and Oettgen, 2010; Dustin, 2009).

B-cell immunity

B-cells constitute around 15 % of peripheral blood leukocytes and are defined by their production of immunoglobulin (Chaplin, 2010). An important function of B cells during an immune response is to produce high affinity antigen-specific (Ag-specific) immunoglobulins (Ig) which facilitate the eradication by phagocytosis of infectious pathogens through efficient opsonisation. Following antigenic stimulation, IgM^+ IgD^+ naïve B cells can undergo isotype switching to produce IgG, IgA or IgE antibodies while retaining their antigen specificity (Snapper et al., 1997).

B cells obtain help from T cells by acting as antigen-specific APCs. Antigen recognition is sequential rather than simultaneous: first, the B-cell binds antigen with its antigen receptor membrane Ig and internalizes and degrades the antigen. Subsequently it presents peptides from the antigen on the cell surface bound class

II MHC molecules. Finally the T cell recognizes the processed antigen on the B cell surface and mutual activation results (Parker, 1993). Each Ig has two identical antigen-binding sites. The carboxyl terminal portions of the heavy and light chains are constant in each subclass of antibody. The heavy chain constant regions pair to form the Fc domain of the molecule that is responsible for most of the effector functions of the Ig molecule, including the binding of Fc receptors on macrophages and the activation of the complement system (Hoebe et al., 2004).

B-cell activation

Mature B cells recirculate through secondary lymphoid organs, including lymph nodes, the spleen and mucosal-associated lymphoid tissues. In the lymph nodes, B cells are concentrated in the cortex in primary follicles in contact with follicular dendritic cells. T cells are in the paracortical areas. Low-molecular-weight antigens might diffuse directly into B-cell areas in secondary lymphoid tissues (Batista and Harwood, 2009). Antigens complexed to varying degrees with IgM, IgG, and complement might be carried on the surfaces of specialized macrophages, follicular dendritic cells, or even B-cells themselves, all of which have receptors for IgG Fc and complement fragments. Antigen presented on these surfaces can stimulate B cells through Ig receptor cross-linking, expression of other interacting surface molecules, and cytokine secretion. B cells require two principle types of signals to become activated.

B-cells are activated as APCs and express peptides along with MHC class II on their surface (Pulendran et al., 2001). These peptides can arise from processed antigen that was internalized after binging on to the B-cell surface immunoglobulin receptor. When the B-cell contacts a $CD4^+$ T cell specific for the combination of such a peptide with self-MHC class II and that has been previously activated by an APC, the $CD4^+$ T cell is able to provide cognate help and activate the B cell for further differentiation into memory cells or plasma cells (König et al., 1996). The activated B cells enters one of two pathways, *i.e.* they either immediately become short-lived plasma cells secreting low-affinity antibodies without somatic mutation, or they enter a follicle to establish a germinal center (Allen et al., 2007). In the germinal center, B cells undergo class-switching (Stavnezer et al., 2008).

At the same time that class-switching is occurring, a mechanism of nucleotide substitution is activated, leading to the accumulation of point mutations in the immunoglobulin heavy and light chains of the variable regions, in a process known as SHM (Peled et al., 2008; Steele, 2009). Also here, the enzymes AID and UNG are important for the DNA cutting and splicing event of class-switching, as well as for the nucleotide substitutions leading to SHM (Peled et al., 2008).

The immune response to the first exposure to an antigen is called the primary response. It is relatively slow, taking a few weeks to develop fully, and leads to production of predominantly IgM antibody of relatively low affinity. Other isotypes,

such as IgG, IgA or IgE, appear relatively late (often more than two weeks) and show higher affinity (affinity maturation). During the primary response, memory T cells and B cells are generated. In a subsequent exposure to the same antigens, these cells are activated more quickly in comparison with a primary response, so that production of high-affinity IgG (or IgA or IgE) is established within one week. This is called a secondary response (Bonilla and Oettgen, 2010; Murphy et al., 2008).

Immune cell communication: the language of chemokines and cytokines

Cells of the immune system require communication networks that can, as required, act locally or at a distance, specifically or globally, and transiently or in a sustained manner. This immune cell communication is conducted mainly by cytokines and chemokines. The term *cytokine* defines a large group of non-enzymatic proteins whose actions are both diverse and overlapping and which affect diverse and overlapping target cell populations (Kelso, 1998; Opals and DePalo, 2000). Chemokines on the other hand are essential for the trafficking of immune effector cells to sites of infection. Moreover, their function is necessary to translate an innate immune response into an adaptive response. Innate immune stimuli, through activation of TLR, set in motion a genetic program that induces the expression of a subset of chemokines from resident tissue macrophages and dendritic cells, and modulates the expression of chemokine receptors on dendritic cells (Luster, 2002; Nomiyama et al., 2010).

Chemokines

Chemokines are small heparine-binding proteins that form a family of chemotactic cytokines that regulate migration and tissue localization of various kinds of cells in the body (Charo and Ransohoff, 2006; Moser et al., 2004; Zlotnik and Yoshie, 2000). In particular, they participate in inflammatory leukocyte recruitment, in lymphocyte recirculation and homing, and even in cancer metastasis (Ben-Baruch, 2008; Gerard and Rollins, 2001). Chemokines have a well conserved region of four cysteines and are grouped into five subfamilies, CXC, CC, XC, CX_3C and CX, based on the arrangement of the two N-terminal cysteine residues (Table 2. 3) (Nomiyama et al., 2010). A single chemokine can bind to several chemokine receptors, whereas a single chemokine receptor can have multiple chemokine ligands (Zlotnik and Yoshie, 2012). The recognition of chemokine-encoded messages is mediated by specific cell-surface G-protein-coupled receptors (GPCRs) with seven transmembrane domains (Murphy, 2002).

Infectious microorganisms can directly stimulate chemokine production by tissue dendritic cells (DCs) and macrophages as well as by many parenchymal and stromal cells. Conserved microbial PAMPs induce chemokines through pattern recognition receptors, such as TLRs, or NOD1 and NOD2 (Girardin et al., 2003; Janeway and Medzhitov, 2002). Classically the major inflammatory and immunomodulatory cytokines such as IL1, TNFα, IFNγ, IL4, IL5, IL6, IL13, and IL17, induced in injury

or infection, stimulate through their respective receptors the production of many different chemokines (Baggiolini et al., 1997; Luster, 1998; Rollins, 1997).

Cytokines

Cytokines are local mediators produced by cells of the lymphoid and macrophage lineage as well as by epithelial and mesenchymal cells. Cytokines are involved in a variety of biological processes, including cell activation, growth, and differentiation, and they are central to the development of inflammation and immunity (Elson, 1996; Sartor, 1994). Cells of the innate immune system, such as macrophages and monocytes, are able to mount a rapid response to a danger signal, *e.g.* an infectious agent, by secreting several pro-inflammatory cytokines such as interleukin (IL)1, IL6, IL12, and tumor necrosis factor (TNF)-α. The cytokine milieu subsequently directs the development of adaptive immunity mediated by T and B lymphocytes (Papadakis and Targan, 2000). The typical cytokine is a glycosylated monomeric peptide of about 150 amino acids (Kelso, 1998). The cytokines are not, however, members of a single gene superfamily. Remarkably few similarities have been noted in their primary nucleotide or amino acid sequences and their genes are, for the most part, scattered throughout the genome (Dinarello, 2000). Currently, there are 18 cytokines with the name interleukin (IL) (Cannon, 2000). Some cytokines clearly promote inflammation and are called pro-inflammatory cytokines, whereas other cytokines suppress the activity of pro-inflammatory cytokines and are called anti-inflammatory cytokines (Dinarello, 2000).

The concept that some cytokines function primarily to induce inflammation while others suppress inflammation is fundamental to cytokine biology and also to clinical medicine (Dinarello, 2000; Opals and DePalo, 2000). A dynamic and ever-shifting balance exists between pro-inflammatory cytokines and anti-inflammatory components of the human immune system. The regulation of inflammation by these cytokines and cytokine inhibitors is complicated by the fact that the immune system has redundant pathways with multiple elements having similar physiologic effects (Kasai et al., 1997; Munoz et al., 1991). The net effect of any cytokine is dependent on the timing of cytokine release, the local milieu in which it acts, the presence of competing or synergistic elements, cytokine receptor density, and tissue responsiveness to each cytokine (Cannon, 2000; Dinarello, 1998). Different immunogens induce the synthesis of different cytokines, which in turn activate different immune effector mechanisms. Although every nucleated cell type can produce cytokines, most lineages express only a subset of cytokine genes (Cannon, 2000; Kelso, 1998).

Within a given lineage, there are several critical levels of control of cytokine synthesis. One is the inducing signal. Cytokines are generally not produced constitutively but the nature of the stimuli that can trigger synthesis varies with the cell type and its differentiation or activation state, and determines which cytokines are produced by that cell. Among the most important stimuli are signals

recognizing either specifically or non-specifically as non self: antigen or antigen-MHC complexes acting via clonotypic receptors on B or T cells; antigen-antibody complexes acting via FC and complement receptors on various lymphoid and inflammatory cell types; superantigens (such as bacterial endotoxins) acting via non-polymorphic regions of certain TCR Vß chains; and other constituents of micro-organisms (Kelso, 1998; Thomson and Lotze, 2003). Once cytokine genes become amenable to transcriptional activation, there are two major levels at which synthesis of cytokines is regulated: transcription rate and mRNA turnover. This has the practical consequence that cytokine mRNA levels are a valid guide to protein production levels in many situations (Akira and Kishimoto, 1997; Jain et al., 1995; Rao et al., 1997; Smale, 2014). Most cytokines are synthesized with a conventional signal sequence that results in their translocation into the Golgi apparatus and rapid secretion from the cell. There are, however, several translational and post-translational levels at which production and release of some cytokines are regulated.

Cytokines deliver signals to target cells via membrane-spanning receptors (Dinarello, 1994; Nicola and Metcalf, 1988). Most cytokine receptors comprise two or more ligand-binding polypeptide chains and, in many cases, one or more of these chains is shared with the receptor for another cytokine. A consequence of this multimeric structure is that most cytokine receptors can exist in two or more affinity states, depending on the availability of individual receptor chains to join the complex (Karnitz and Abraham, 1996; Sugamura et al., 1996). Ligand-induced cross-linking of cytokine receptor chains causes interaction of their associated Janus kinases (JAKs). It is thought that the Jaks first phosphorylate each other and thereafter phosphorylate sites in the cytoplasmic receptor domains (Yamaoka et al., 2004). The latter events enable Signal Transducer and Activator of Transcription (STAT) binding and phosphorylation, as well as triggering the Ras/MAP kinase and phosphatidylinositol cascades (Karnitz and Abraham, 1996). Activation of the bound STAT molecules causes homo- or heterodimerization to another STAT molecule. These STAT dimers then translocate to the nucleus where they bind to the regulatory regions of the relevant cytokine-responsive genes to initiate transcription (Kelso, 1998). In general, specificity in cytokine receptor signaling appears to be achieved through the association between the receptor itself and a particular STAT, rather than via the JAKs (Kaplan et al., 1996a, 1996b; Kopf et al., 1993; Kuhn et al., 1991).

Here we will discuss some, *i.e.,* IL1, IL2, IL6, IL10 and TGFß, but not all interleukins. The IL1 cytokine family comprises four main members IL1α, ILß, IL1 receptor antagonist (IL1ra/IL1RN) and IL18 (Girn et al., 2007). The IL1 family is primarily considered to be pro-inflammatory, as it can up-regulate host defenses and act as an immunoadjuvant (Dinarello, 1997a). IL1ß plays a significant role in inflammation, as it has been implicated in enhancing expression of cell adhesion molecules on the endothelial surface and has consequently been deemed to be pro-

atherogenic (Dinarello, 1999). The only member of this family with paradoxical properties is IL1RN, a naturally occurring cytokine antagonist, which plays an anti-inflammatory role in regulating IL1 function (Dinarello and Thompson, 1991; Perrier et al., 2006). IL1RN blocks the action of IL1α and IL1ß functional ligands by competitive inhibition at the IL1 receptor level (Dinarello, 1997b; Sims et al., 1993). After attachment of IL1 to its receptor, intracellular signaling occurs after a heterodimeric complex is formed between the IL1 receptor and an essential second protein known as IL1 receptor-accessory protein (Greenfeder et al., 1995). IL1RN will bind with high affinity to the IL1 receptor but fails to engage the IL1 receptor accessory protein. This occupies the membrane-bound IL1 receptor binding site and prevents cellular activation by IL1α or IL1ß by steric inhibition (Schreuder et al., 1997). The anti-inflammatory cytokines IL4, IL6, IL10 and IL13 inhibit the synthesis of IL1ß and stimulate the synthesis of IL1RN (Dinarello, 1997b).

The IL2 family comprises four representative members: IL2, IL4, IL5 and granulocyte-monocyte colony stimulating factor (GM-CSF). The most noteworthy action of IL2 relates to its mitogenic effects on T lymphocytes in response to antigenic stimulation, including the generation of both cytotoxic and suppressor T cells (Girn et al., 2007). IL4 is a highly pleiotropic cytokine that is able to influence T_H cell differentiation. Early secretion of IL4 leads to polarization of T_H cell differentiation towards T_H2-like cells (Mosmann et al., 1986), which secrete their own IL4, and subsequently autocrine production of IL4 supports cell proliferation. The T_H2 cell secretion of IL4 and IL10 leads to the suppression of T_H1 responses by down-regulating the production of macrophage-derived IL12 and inhibiting the differentiation of T_H1-type cells (Brown and Hural, 1997; Kelso, 1995; Mosmann et al., 1986). IL4 drives T_H2 responses, mediates the recruitment and activation of mast cells, and stimulates the production of IgE antibodies via the differentiation of B cells into IgE-secreting cells (Brown and Hural, 1997; Wang et al., 1995). IL4 has marked inhibitory effects on the expression and release of the pro-inflammatory cytokines. It is able to block or suppress the monocyte-derived cytokines including IL1, TNFα, IL6, IL8 and macrophage inflammatory protein (MIP)-1α (Brown and Hural, 1997; Paul, 1991; te Velde et al., 1990; Wang et al., 1995). It has also been shown to suppress macrophage cytotoxic activity, parasite killing and macrophage-derived nitric oxide production (Vannier et al., 1992). In addition to its inhibitory effects on the production of pro-inflammatory cytokines, it stimulates the synthesis of the cytokine inhibitor IL1RN (Dinarello, 1997b; Hart et al., 1989).

IL6 has long been regarded as a pro-inflammatory cytokine induced by LPS along with TNFα and IL1. It is often used as a marker for systemic activation of pro-inflammatory cytokines (Barton and Medzhitov, 2002). Like many other cytokines, IL6 has both pro- and anti-inflammatory properties. Although IL6 is a potent inducer of the acute-phase protein response, it has anti-inflammatory properties as well (Barton et al., 1996). After binding to its specific α receptor, IL6 complexes with the ubiquitous gp130 signal transducing unit. IL6 belongs to a family of gp130

receptor ligands that includes IL11, leukemia inhibitory factor, ciliary neurotrophic factor, oncostatin M, and cardiotrophin-1. IL6 down-regulates the synthesis of IL1 and TNFα (Libert et al., 1994; McGeough et al., 2012). IL6 attenuates the synthesis of the pro-inflammatory cytokines while having little effect on the synthesis of anti-inflammatory cytokines such as IL10 and transforming growth factor ß (TGFß). IL6 induces the synthesis of glucocorticoids and promotes the synthesis of IL1RN and soluble TNF receptor release in human volunteers (Ruzek et al., 1997; Tilg et al., 1994). At the same time, IL6 inhibits the production of pro-inflammatory cytokines such as GM-CSF, IFNγ and MIP-2 (Barton, 1997).

IL10 is the most important anti-inflammatory cytokine produced by a variety of cells, including T and B lymphocytes, thymocytes, macrophages, mast cells, keratinocytes and intestinal epithelial cells (Opals and DePalo, 2000). It is a potent inhibitor of T_H1 cytokines, including both IL2 and IFNγ, but also of IL1, IL6 and TNFα (Hagenbaugh et al., 1997; Howard and O'Garra, 1992; Lalani et al., 1997; Opal et al., 1998). IL10 is also a potent deactivator of monocyte/macrophage pro-inflammatory cytokine synthesis (Brandtzaeg et al., 1996; Clarke et al., 1998). It also inhibits cell surface expression of MHC class II molecules and the LPS recognition and signaling molecule CD14 (Opal et al., 1998).

TGFß is synthesized as an inactive precursor and requires activation before exerting its effects (Nørgaard et al., 1995). It is an important regulator of cell proliferation, differentiation, and formulation of the extracellular matrix (Letterio and Roberts, 1997). Like many cytokines (*e.g.* IL6), TGFß has both pro- and anti-inflammatory effects. It functions as a biological switch, antagonizing or modifying the action of other cytokines and growth factors. The presence of other cytokines may modulate the cellular responses of TGFß, and the effect may differ depending on the activation state of the cell (Kingsley, 1994). TGFß is capable of converting an active site of inflammation into one dominated by resolution and repair (Letterio and Roberts, 1997).

The cytokine induced immune responses can be further regulated by suppressors of cytokine signaling (SOCS) and the cytokine-inducible SH2 protein (CIS) family of intracellular proteins (Alexander, 2002; Greenhalgh et al., 2002; Yasukawa et al., 2000). In total there are eight SOCS proteins (*i.e.* SOCS1, SOCS2, SOCS3, SOCS4, SOCS5, SOCS6, SOCS7 and CIS), each of which has a central SH2 domain, an amino-terminal domain of variable length and divergent sequence, and a carboxy-terminal 40-amino-acid module that is known as the SOCS box (Illson et al., 1998). The function of the SOCS box is the recruitment of the ubiquitin-transferase system. The SOCS box interacts with elongins B and C, cullins, Rbx-1 and E2 (Kamura et al., 1998; Zhang et al., 1999b). Thus, CIS and SOCS family proteins, as well as other SOCS box-containing molecules, probably function as an E3 ubiquitin ligase and mediate the degradation of proteins associated through their N-terminal regions. However, the SOCS box is also important for the stabilization and/or degradation of the SOCS1 and SOCS3 proteins themselves (Kamura et al., 1998).

Interaction of the SOCS box with elongin C stabilizes SOCS3 protein expression, whereas phosphorylation of SOCS box tyrosine residues disrupts the complex and enhances proteasome-mediated degradation of SOCS3 (Haan et al., 2003).

The best characterized SOCS-family members, SOCS1, SOCS2, SOCS3 and CIS (Table 2. 4), seem to act in a classical negative-feedback loop to inhibit cytokine signal transduction (Alexander, 2002). CIS and SOCS2 bind to phosphorylated tyrosine residues on activated (phosphorylated) cytokine receptors. They also compete with STATs or can sterically hinder the STAT binding sites of receptors, inhibiting STAT activation, as in the case of STAT5 (Ram and Waxman, 1999; Yoshimura et al., 2007). CIS is induced by cytokines that activate STAT5, such as erythropoietin, IL2, IL3, prolactin and growth hormone (Yoshimura et al., 1995).

SOCS1 has an important regulatory function in macrophages and dendritic cells. The inhibitory activity of SOCS2 is not as strong as that of CIS, and SOCS2 seems to be a relatively specific negative regulator of the growth hormone-STAT5 pathway (Metcalf et al., 2000). Both SOCS1 and SOCS3 can inhibit JAK tyrosine kinase activity. They have a kinase inhibitory region in their N-terminal domain, which probably functions as a pseudosubstrate (Yasukawa et al., 1999). SOCS1 uses its SH2 domain to directly bind the activation loop of JAKs and binds the catalytic pocket of JAKs through its kinase inhibitory region (Giordanetto and Kroemer, 2003).

The mechanism of SOCS3-mediated inhibition of signaling involves both the cytokine receptors and JAKs (Hansen et al., 1999; Nicholson et al., 2000; Sasaki et al., 2000). SOCS3 binds activated receptors but does not seem to interfere with STAT recruitment (Bjørbaek et al., 1998; Cohney et al., 1999; Hansen et al., 1999; Nicholson et al., 2000; Sasaki et al., 2000). Rather, SOCS3 inhibits JAKs catalytic activity in a manner that is analogous to SOCS1, but SOCS3 relies on receptor binding, rather than a direct interaction with JAKs, to gain access to the JAK activation loop. Moreover, SOCS3 binds the GP130 receptor at a specific phosphorylated receptor tyrosine that is the same as that used by the signaling molecule SH2-domain-containing protein tyrosine phosphatase 2 (SHP2) for receptor interaction (Nicholson et al., 2000; Schmitz et al., 2000). As SH2P can promote GP130 signaling through the activation of mitogen-activated protein kinases, it is possible that SOCS3 will suppress aspects of GP130 signaling also by competing with SHP2 for receptor binding. Thus SOCS3 selectively blocks IL6 signaling, interfering with the ability of the latter to inhibit LPS signaling (Kubo et al., 2003).

Table 2. 3: CC, CXC, CX_3C and XC families of chemokines and chemokine receptors.

Receptor	Chemokine ligands	Cell types	Reference
CCR1	CCL3, CCL5, CCL7, CCL14	T cells, monocytes, eosinophils, basophils	Mahad *et al*. (2004); Proudfoot *et al*. (2003); Trebst *et al*. (2001)
CCR2	CCL2, CCL8, CCL7, CCL13, CCL16	Monocytes, dendritic cells (immature), memory T cells	Charo (2004); Charo and Peters (2003); Gu *et al*. (2000); Proudfoot *et al*. (2003)
CCR3	CCL11, CCL13, CCL7, CCL5, CCL8,	Eosinophils, basophils, mast cells, T_H2 cells, platelets	Daly and Rollins (2003)
CCR4	CCL17, CCL22	T cells (T_H2), dendritic cells (mature), basophils, macrophages, platelets	Calzascia *et al*. (2005); Flier *et al*. (2001)
CCR5	CCL3, CCL4, CCL5, CCL11, CCL14,	T cells, monocytes	Mahad *et al*. (2004); Proudfoot *et al*. (2003); Trebst *et al*. (2001)
CCR6	CCL20	T cells (T regulatory and memory), B cells, dendritic cells	Schutyser *et al*. (2003)
CCR7	CCL19, CCL21	T cells, dendritic cells (mature)	Cyster (2003, 1999); Mantovani (1999); Sozzani *et al*. (2000)
CCR8	CCL1	T cells (T_H2), monocytes, dendritic cells	Qu *et al*. (2004)
CCR9	CCL25	T cells, IgA^+ plasma cells	Calzascia *et al*. (2005)
CCR10	CCL27, CCL28	T cells	Homey *et al*. (2002); Wang *et al*. (2000)

CXCR1	CXCL8 (IL-8), CXCL6	Neutrophils, monocytes	Gerszten *et al*. (1999); Liehn *et al*. (2013); Tachibana *et al*. (1998)
CXCR2	CXCL8, CXCL1, CXCL2, CXCL3, CXCL5, CXCL6	Neutrophils, monocytes, micro-vascular endothelial cells	Gerszten *et al*. (1999); Liehn *et al*. (2013); Tachibana *et al*. (1998)
CXCR3-A	CXCL9, CXCL10, CXCL11	Type 1 helper cells, mast cells, mesangial cells	Flier *et al*. (2001); Sørensen *et al*. (1999)
CXCR3-B	CXCL4, CXCL9, CXCL10, CXCL11	Microvascular endothelial cells, neoplastic cells	Flier *et al*. (2001); Sørensen *et al*. (1999)
CXCR4	CXCL12	Widely expressed	Ma *et al*. (1998); Zou *et al*. (1998)
CXCR5	CXCL13	B cells, follicular helper T cells	Cyster *et al*. (1999); Müller *et al*. (2003)
CXCR6	CXCL16	$CD8^+$ T cells, natural killer cells, memory $CD4^+$ T cells	Matloubian *et al*. (2000); Shimaoka *et al*. (2000)
CX3CR1	CX3CL1	Macrophages, endothelial cells, smooth-muscle cells	Bazan et al. (1997); Pan et al. (1997)
XCR1	XCL1, XCL2	T-cells, natural killer cells	Kelner et al. (1994)

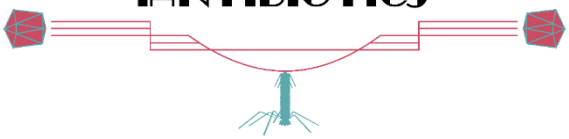
Table 2. 4: Cytokine induction and activation of SOCS proteins. Adapted from *Alexander (2002) and Alexander and Hilton (2004).*

SOCS protein	Induced by	Inhibits	Reference
CIS	IL-2, IL-3, IL-6, IL-9, IL-10, GM-CSF, GH, PRL, TSLP, EGF, CNTF, leptin, EPO, TPO	IL-2, IL-3, GH, IGF1, leptin, EPO	Adams et al. (1998); Aman et al. (1999); Bjørbæk et al. (1999); Emilsson et al. (1999); Isaksen et al. (1999); Lejeune et al. (2001); Okabe et al. (1999); Pezet et al. (1999); Ram and Waxman (1999); Sadowski et al. (2001); Shen et al. (2000); Starr et al. (1997); Yoshimura et al. (1995); Zong et al. (2000)
SOCS1	IL-2, IL-4, IL-6, IL-9, IL-10, G-CSF, GH, PRL, TSH, SCF, insulin, LIF, CT1, CNTF, EPO, IFN-α/β, IFN-γ, TNF	IL-2, IL-3, IL-4, IL-6, IL-7, M-CSF, GH, IGF1, PRL, TSLP, SCF, Flk ligand, insulin, LIF, OSM, CT1, EPO, TPO	Adams et al. (1998); Bjørbæk et al. (1999); Bourette et al. (2001); De Sepulveda et al. (1999); Endo et al. (1997); Hamanaka et al. (2001); Isaksen et al. (1999); Kawazoe et al. (2001); Lejeune et al. (2001); Losman et al. (1999); Morita et al. (2000); Naka et al. (1997); Park et al. (2000); Pezet et al. (1999); Sadowski et al. (2001); Shen et al. (2000); Song and Shuai (1998); Sporri et al. (2001); Starr et al. (1997); Trop (2001); Wang et al. (2000); Zong et al. (2000)
SOCS2	IL-2, IL-6, GH, PRL, insulin, CNTF	GH, IGF1, LIF	(Adams et al. (1998); Bjørbæk et al. (1999); Minamoto et al. (1997); Pezet et al. (1999); Ram and Waxman (1999); Sadowski et al. (2001); Starr et al. (1997); Zong et al. (2000)
SOCS3	IL-1, IL-2, IL-3, IL6, IL-9, IL-10, IL-11, Il-22, GH, PRL, TSH, EGF, insulin, PDGF, BFGF, LIF, OSM, CT1, CNTF, leptin, EPO, TPO, TNF	IL-1, IL-2, IL-3, IL-4, IL-6, IL-9, IL-11, GH, IGF1, PRL, insulin, LIF, OSM, CT1, CNTF, leptin, EPO, INF-α/β, INF-γ	Adams et al. (1998); Auernhammer et al. (1998); Auernhammer and Melmed (1999); Bjørbaek et al. (1998); Bjørbæk et al. (1999); Boisclair et al. (2000); Cacalano et al. (2001); Cohney et al. (1999); Emanuelli et al. (2000); Hamanaka et al. (2001); Hong et al. (2001); Karlsen et al. (2001); Kotenko et al. (2001); Lejeune et al. (2001); Losman et al. (1999); Magrangeas et al. (2001a, 2001b); Minamoto et al. (1997); Nicholson et al. (1999); Park et al. (2000); Pezet et al. (1999); Sadowski et al. (2001); Sasaki et al. (2000); Shen et al. (2000); Song and Shuai (1998); Starr et al. (1997); Terstegent et al. (2000); Wang et al. (2000); Zong et al. (2000)

BFGF: basic fibroblast growth factor; CIS: cytokine-induced SRC-homology-2 protein; CNTF: ciliary neurotrophic factor; CT1: cardiotrophin-1; EGF: epidermal growth factor; EPO: erythropoietin; G-CSF: granulocyte colony-stimulating factor; GH: growth hormone; GM-CSF: granulocyte-macrophage colony-stimulating factor; IFN: interferon; IGF1: insulin-like growth factor-1; IL: interleukin; LIF: leukemia inhibitory factor; M-CSF: macrophage colony-stimulating factor; OSM: oncostatin M; PDGF: platelet-derived growth factor; PRL: prolactin; stem-cell factor; SOCS: suppressor of cytokine signaling; TNF: tumor necrosis factor; TPO thrombopoietin; TSH: thyrotropin; TSLP: thymic stromal lymphopoietin.

Chapter 3

ANTIBIOTICS



"Penicillin is to all intents and purposes non-poisonous so there is no need to worry about giving an overdose and poisoning the patient. There may be a danger, though, in underdosage. It is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them, and the same thing has occasionally happened in the body."

- Fleming, Nobel lecture, 1945

Introduction - the antibiotics resistance problem

The introduction of antibiotics is one of the most important medical interventions with regard to reducing human morbidity and mortality. However, the intensive use of antibiotics has dramatically increased the frequency of resistance among human pathogens and threatens a loss of therapeutic options and a post-antibiotic era in which the medical advances are no longer effective (Guay, 2008; Lew et al., 2008; Woodford and Livermore, 2009). The clinical use of antibiotics, and therefore the effective treatment of bacterial infections, is under considerable threat due to the emergence of bacteria that are resistant to many classes of commonly used antibiotics. In the traditional sense, antibiotic resistance is often considered to be a trait acquired by previously susceptible bacteria, the basis of which can be attributed to the horizontal acquisition of new genes, or the occurrence of spontaneous mutations within chromosomally located genes that are subsequently transmitted vertically as the bacteria replicate (Davies, 2007; Martinez and Baquero, 2000). In addition to the ability of bacteria to 'acquire' resistance, different bacterial species are also intrinsically resistant to different classes of antibiotics (Cox and Wright, 2013).

The accumulation of antibiotic resistance in bacteria provides the most drastic demonstration of Darwinian selection available to us, and one with serious practical consequences (Livermore, 2007). Antibiotics select for those bacteria that are inherently resistant or that have acquired resistance via mutation or DNA transfer. Upon the introduction of antibiotics, it was assumed that the development of antibiotic resistance was unlikely, although addressed by Alexander Flemming in his nobel prize acceptance speech (Fleming, 1945). This was based on the assumption that the frequency of mutations generating resistant bacteria was neglectable (Davies, 1994). Bacterial mutation rates in the laboratory are usually $< 10^{-7}$ and are often $\leq 10^{-9}$, whilst plasmid transfer rates are generally $< 10^{-5}$ per donor cell, but these apparently low rates must be multiplied by the huge numbers of bacteria exposed. The human gut is estimated to contain $10^{13} – 10^{14}$ bacteria, tenfold more than the total amount of human cells in the body, and these are exposed to selection pressure whenever antibiotics are used (Guarner and Malagelada, 2003). Moreover, antibiotics are not only used in human medicine but have also been applied for treatment, mass prophylaxis and growth promotion in animals, with resistant bacteria passed to humans via the food chain (Livermore, 2007).

Antibiotic resistance mechanisms

To understand how antibiotics work, and subsequently how antibiotic resistance can develop, insights regarding the targets of the main classes of these antibacterial drugs is required: bacterial cell-wall biosynthesis, bacterial protein synthesis and bacterial DNA replication and repair (Table 3. 1) (Walsh, 2000).

Bacterial resistance to antibiotics can be intrinsic or innate, which is characteristic for a particular bacterium and depends on the biology of the microorganism, or can be acquired (Alanis, 2005; Giedraitienė et al., 2011). Resistance of Mollicutes (best

known genus is *Mycoplasma*) to ß-lactam antibiotics is an example of intrinsic resistance, explained by the lack of a cell wall in this class of bacteria (Bébéar and Pereyre, 2005). Acquired resistance occurs from acquisition of exogenous genes by uptake of free DNA (transformation) plasmids (conjugation), transposons (conjugation), integrons and bacteriophages (transduction), mutation or a combination of these mechanisms (Hawkey, 1998). The main types of biochemical mechanisms that bacteria use for defense against antibiotics are enzymatic inactivation or modification, decreased uptake or efflux, and alteration or overproduction of the target (Chen et al., 2011; Wright, 2005).

Clinically relevant drug resistant strains

Pseudomonas aeruginosa

Pathogenicity

Pseudomonas aeruginosa is an important bacterial pathogen, particularly as cause of infections in hospitalized patients and immunocompromised hosts such as burn wound patients and patients witsh cystic fibrosis. *P. aeruginosa* is a Gram-negative aerobic opportunistic bacterium that normally inhabits the soil and surfaces in aqueous environments (Gellatly and Hancock, 2013). It is an important cause of both community- and hospital-acquired infections. Community-acquired infections include, but are not limited to, ulcerative keratitis (usually associated with contact lens use), otitis externa (typically in immunocompromised hosts such as those with diabetes mellitus), and skin and soft tissue infections (including diabetic foot infections and infections of burn wounds) (Driscoll et al., 2007). Hospitalized patients may be colonized with *P. aeruginosa* or may acquire it during their hospital stay. *P. aeruginosa* can be isolated from nearly any conceivable source within hospitals (Bonten et al., 1999; Pirnay et al., 2003). Nosocomial infections caused by *P. aeruginosa* include pneumonias, urinary tract infections, bloodstream infections, surgical site infections and skin infections in the setting of burn injuries. Infections with *P. aeruginosa* are not only common, they also have been associated with high morbidity and mortality when compared with other bacterial pathogens (Harbarth et al., 2002; Kollef et al., 2005; Osmon et al., 2004; Rello et al., 2002).

P. aeruginosa has been identified as the second most common cause of hospital-acquired pneumonia (HAP), healthcare-associated pneumonia (HCAP) and ventilator-associated pneumonia (VAP), exceeded in frequency only by *Staphylococcus aureus* (Gaynes et al., 2005; Kollef et al., 2005). *P. aeruginosa* is often identified as the most frequent infectious isolate in burn units, and accounts for a large percentage of documented wound infections, bacteremia and VAP in these units (Lari and Alaghehbandan, 2000; Sewunet et al., 2013; Song et al., 2001). *P. aeruginosa* also plays an important role in patients with cystic fibrosis (CF), in whom chronic and recurrent infections of the sinopulmonary tract by *P. aeruginosa* are common.

P. aeruginosa possesses a single flagellum that enables motility and that may mediate initial surface interactions (O'Toole and Kolter, 1998). *P. aeruginosa* also has multiple cell surface pili (type IV) that are responsible for adherence to cell membranes and other surfaces (Kipnis et al., 2006; Lau et al., 2005). During an infection, the bacterium can adhere to host epithelial cells through the binding of its flagellum to the asialyated glycolipid asialoGM1 and can elicit a strong NF-κB-mediated inflammatory response via signaling through TLR5 and a caspase-1-mediated response through the NLR Ipaf (Miao et al., 2007). Type IV pili are the most important adhesion molecules of *P. aeruginosa* and are also involved in twitching motility and the formation of biofilms (Kipnis et al., 2006). Pili can also lead to aggregation, causing the bacteria to form microcolonies on target tissues, effectively concentrating the bacteria in one location and potentially offering protection from the host immune system and from antibiotics (Craig et al., 2004; Sriramulu et al., 2005). Some isolates of *P. aeruginosa* overproduce the extracellular polysaccharide alginate (a condition called mucoidy), with associated mucoid morphology apparent on culture (Lau et al., 2005). Alginate has been noted to have a number of effects that may impede bacterial clearance by the infected host, including scavenging of free radicals released by macrophages, providing a physical barrier that impairs phagocytosis, and inhibiting neutrophil chemotaxis and complement activation (Ramsey and Wozniak, 2005).

Many pathogenic Gram-negative bacteria, such as *Yersinia*, *Salmonella*, *Shigella* and *Pseudomonas* species share a type three secretion system (T3SS) as a means of injecting toxins directly into host cells (Kipnis et al., 2006). As such, the *P. aeruginosa* T3SS is a major determinant of virulence, and its expression is frequently associated with acute invasive infections and has been linked to increased mortality in infected patients (Hauser, 2009; Sadikot et al., 2005). The needle-like appendage of the T3SS, evolutionarily related to flagella, permits the translocation of effector proteins from the bacterium into the host cell through a pore formed in the host cell membrane (Hauser, 2009). There are four known toxins, variably expressed in different strains and isolates, injected into host cells by *P. aeruginosa* through the T3SS: ExoS, ExoT, ExoY and ExoU (Gellatly and Hancock, 2013; Kipnis et al., 2006).

ExoS is a bifunctional cytotoxin with two active domains, a C-terminal ADP-ribosyltransferase domain and an N-terminal Rho GTPase-activating protein (GAP) domain (Fu et al., 1993). The pathogenic role of ExoS is mainly attributable to the ADP-ribosyltransferase activity leading to disruption of normal cytoskeletal organization (Maresso et al., 2004; Shaver and Hauser, 2004). The C-terminal domain binds to TLR2 and the N-terminal domain binds to TLR4, showing that ExoS may also modulate the host immune and inflammatory response (Epelman et al., 2004). ExoT is similar to ExoS, with dual ADP-ribosyltransferase and GAP activities, although the ExoT ADP-ribosyltransferase targets different pathways (Aktories and Barbieri, 2005; Henriksson et al., 2002). ExoY is an adenylate cyclase injected directly into the host cytosol by the T3SS and increases cytosolic cyclic AMP

(cAMP), enhanced by a eukaryotic cofactor (Yahr et al., 1998). This increased cytosolic cAMP leads to increased pulmonary microvascular intercellular gap formation and increased lung permeability (Sayner et al., 2004). ExoU was recently found to have a phospholipase/lysophospholipase activity disrupting eukaryotic cell membranes after translocation into the cell by the T3SS and activation by a yet unknown eukaryotic cofactor (Pankhaniya et al., 2004; Sato et al., 2003; Tamura et al., 2004).

Besides these four toxins, *P. aeruginosa* is able to secrete additional virulence factors, such as pyocyanin and pyoverdine, proteases, phospholipase C and exotoxin A (ExoA). Pyocyanin is a blue pigment metabolite of *P. aeruginosa* that has been shown to have numerous pathogenic effects such as an increase in IL8, depression of host-response, and induction of apoptosis in neutrophils (Allen et al., 2005; Denning et al., 1998; Leidal et al., 2001). Pyoverdine is a siderophore, a small molecule chelating iron from the environment for use in *P. aeruginosa* metabolism (Meyer et al., 1996; Takase et al., 2000). Phospholipase C, more specifically hemolytic phospholipase C, is a phospholipase secreted by *P. aeruginosa* into the extracellular space through a type two secretion system (T2SS). It targets eukaryotic membrane phospholipids (König et al., 1997; Wiener-Kronish et al., 1993). ExoA, secreted into the extracellular space through a T2SS, is an ADP-ribosyltransferase inhibiting elongation factor-2 (EF-2), thereby inhibiting protein synthesis and leading to cell death (Pavlovskis et al., 1978; Wick et al., 1990).

Another major virulence factor of *P. aeruginosa* are its lipopolysaccharides. Lipopolysaccharide is a complex glycolipid that forms the outer leaflet of the outer membrane of Gram-negative bacteria and has roles in antigenicity, the inflammatory response, exclusion of external molecules, and in mediating interactions with antibiotics (King et al., 2009). *P. aeruginosa* produces a three-domain lipopolysaccharide consisting of a membrane-anchored lipid A, a polysaccharide core region, and a highly variable O-specific polysaccharide (Gellatly and Hancock, 2013). The production, function and virulence of lipopolysaccharides are discussed more thoroughly in **Chapter 4 - Endotoxins**.

Antibiotic resistance

Surveillance of *P. aeruginosa* isolated from hospitalized patients has revealed alarming antimicrobial resistance increase (Driscoll et al., 2007; Solomon et al., 2002). Infections by *P. aeruginosa* are notoriously difficult to treat due to its intrinsic ability to acquire resistance. All known mechanisms of antibiotic resistance can be displayed by this bacterium (Moore and Flaws, 2011). In the case of *P. aeruginosa,* intrinsic resistance is due to the low permeability of its outer membrane, the constitutive expression of membrane efflux pumps, and the natural occurrence of an inducible ß-lactamase, AmpC (Strateva and Yordanov, 2009).

Table 3. 1: Targets, mode of action and mechanisms of resistance of the main classes of antibacterial drugs. Table adapted from Walsh (2000).

Antibiotic	Target	Mode of action	Resistance mechanism	Reference
Cell-wall				
ß-lactams	Transpeptidases/ transglycosylases (PBPs)	Blockade of the crosslinking enzymes in the peptidoglycan layer of the cell-wall	ß-lactamases, PBP	Spratt and Cromie (1988)
Vancomycin	D-Ala-D-Ala termini of peptidoglycan and lipid II	Sequestration of substrate required for crosslinking	Reprogramming of D-Ala-D-Ala to D-Ala-D-Lac or D-Ala-D-Ser	Arthur and Courvalin, (1993); Walsh *et al.* (1996); Williams (1996)
Protein synthesis				
Macrolides of the erythromycin class	Peptidyl transferase, center	Blockade of protein synthesis	rRNA methylation, drug efflux	Brisson-Noël *et al.* (1988)
Tetracyclines	Peptidyl transferase	Blockade of the protein synthesis	Drug efflux	Chopra and Roberts (2001)
Aminoglycosides	Peptidyl transferase	Blockade of protein synthesis	Enzymatic modification of drug	Fourmy *et al.* (1996)
Oxazolidinones	Peptidyl transferase	Blockade of protein synthesis	Peptidyl transferase	Kloss *et al.* (1999)
DNA replication/repair				
Fluoroquinolones	DNA gyrase	Blockade of DNA replication	Gyrase mutations to drug resistance	Anderson *et al.* (2012)

The outer membrane of Gram-negative bacteria acts as a selective barrier for uptake of antibiotics (Nicas and Hancock, 1983). *P. aeruginosa* has a large exclusion limit owing to the limited number of large channels of its major porin OprF, and the small size of the channels of other porins that mediate the passage of other molecules of the size of antibiotics, including OprD and OprB (Breidenstein et al., 2011). Members of the RND family of efflux pumps appear to be the most significant contributors to antimicrobial resistance of *P. aeruginosa* (Poole, 2007).

P. aeruginosa also has an intrinsic resistance against ß-lactams through the expression of AmpC and PoxB (Girlich et al., 2004; Kong et al., 2005; Lodge et al., 1990). While the original ß-lactamases were plasmid-encoded restricted-spectrum class A enzymes that only hydrolyzed penicillins and older, narrow-spectrum cephalosporins, more recently described acquired ß-lactamases in *P. aeruginosa* include the extended-spectrum ß-lactamase (ESBL) enzymes able to hydrolyze a wider range of ß-lactams, including the carbapenems (Zhao and Hu, 2010).

Multidrug resistant strains of *P. aeruginosa* typically exhibit several resistance mechanisms simultaneously, whereby resistance to specific antibacterials may be mediated by different combinations of these mechanisms (Deplano et al., 2005; Dubois et al., 2001).

Staphylococcus aureus

Pathogenicity

Staphylococcus aureus is both a commensal organism and a pathogen. The anterior nares are the main ecological niche for *S. aureus*. Approximately 20 % of individuals have nares persistently colonized with *S. aureus*, and 30 % are intermittently colonized. However, numerous other sites may be colonized, including the axillae, groin, and gastrointestinal tract (Gordon and Lowy, 2008). Colonization increases the risk for subsequent infection (Kluytmans et al., 1997; Wertheim et al., 2005). Those with *S. aureus* infections are generally infected with their colonizing strain (Williams et al., 1959). Colonization also allows *S. aureus* to be transmitted among individuals in both health care and community settings. The primary mode of transmission of *S. aureus* is by direct contact, usually skin-to-skin contact with a colonized or infected individual, although contact with contaminated objects and surfaces might also have a role (Kazakova et al., 2005; L and K, 2006; Miller and Diep, 2008; Muto et al., 2003).

S. aureus produces a wide variety of exoproteins that contribute to its ability to colonize and to cause disease in mammalian hosts. Nearly all strains secrete a group of enzymes and cytotoxins, which includes four hemolysins (alpha, beta, gamma, and delta), nucleases, proteases, lipases, hyaluronidase, and collagenase. The main function of these proteins may be to convert local host tissues into nutrients required for bacterial growth. Some strains produce one or more additional exoproteins, which include toxic shock syndrome toxin-1 (TSST-1), the staphylococcal enterotoxins (SEA, SEB SEC, SED, SEE, SEG, SEH, and SEI), the

exfoliative toxins (ETA and ETB), and leucocidin (Dinges et al., 2000). Toxic shock syndrome toxin-1 and the staphylococcal enterotoxins are also known as pyrogenic toxin superantigens (PTSAgs) and are secreted by either *S. aureus* or *Streptococcus pyogenes* (Bohach et al., 1990). Each of these exotoxins exhibits at least three biological properties: pyrogenicity, superantigenicity, and the capacity to enhance the lethality of endotoxin in rabbits up to 100,000 fold (Bohach et al., 1990; Marrack and Kappler, 1990). TSST-1 is unique in its ability to cross mucosal surfaces and is the only PTSAg known to reactivate bacterial cell wall-induced arthritis (Hamad et al., 1997; Schwab et al., 1993). The best characterized property of the PTSAgs is superantigenicity, which refers to the ability of these exotoxins to stimulate proliferation of T lymphocytes without regard for the antigen specificity of these cells by linking the TCR and the MHC class II molecule (Fleischer and Schrezenmeier, 1988; Marrack and Kappler, 1990; Rödström et al., 2014; White et al., 1989).

In addition to their functional similarities, the staphylococcal PTSAgs share a number of genetic and biochemical characteristics. Like most proteins secreted by *S. aureus*, they are produced primarily in the postexponential phase of growth (Dinges et al., 2000). Comparison of the three-dimensional structure of TSST-1 to those of SEA, SEB and SEC, it was demonstrated that each of these proteins is folded into a highly prototypical structure. This high level of structural homology is not surprising in view of their functional relatedness. Of considerable interest are the molecular structures of PTSAgs in complex with MHC class II molecules or the ß-chain of the TCR (Hoffmann et al., 1994; Papageorgiou et al., 1999; Prasad et al., 1997; Schad et al., 1995; Swaminathan et al., 1992).

In establishing an infection, *S. aureus* has numerous surface proteins, called microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) that mediate adherence to host tissues. MSCRAMMs bind molecules such as collagen, fibronectin, and fibrinogen, and different MSCRAMMs may adhere to the same host-tissue component (Foster and Höök, 1998; Menzies, 2003; Patti et al., 1994; Tung et al., 2000). Once *S. aureus* adheres to the host tissue or prosthetic materials, it is able to grow and persist in various ways. *S. aureus* can form biofilms on host and prosthetic surfaces, enabling it to persist by evading host defenses and antimicrobials (Donlan and Costerton, 2002). *In vitro*, *S. aureus* can also invade and survive inside epithelial cells, including endothelial cells, which theoretically may also allow it to escape host defenses, particularly in endocarditis (Gordon and Lowy, 2008; Hamill et al., 1986; Moreillon et al., 2002; Ogawa et al., 1985). *S. aureus* may also secrete chemotaxis inhibitory protein of staphylococci or the extracellular adherence protein, which interfere with neutrophil extravasation and chemotaxis to the site of infection (Foster, 2005). The mortality of *S. aureus* bacteremia remains approximately 10 – 30 % despite the availability of effective antimicrobials (van Hal et al., 2012). *S. aureus* is now the leading overall cause of nosocomial infections and, as more patients are treated outside the hospital setting,

is an increasing concern in the community (Diekema et al., 2001; Solomon et al., 2002).

Antibiotic resistance

S. aureus is naturally susceptible to virtually every antibiotic that has ever been developed. Resistance to antibiotics is often acquired by the horizontal transfer of genes from outside sources, although chromosomal mutation is also important (Chambers and Deleo, 2009). As rapidly as new antibiotics have been introduced, staphylococci have developed efficient mechanisms to neutralize them (Table 3. 2).

Staphylococcal resistance to penicillin is mediated by *blaZ*, the gene that encodes ß-lactamase. This predominantly extracellular enzyme, synthesized when staphylococci are exposed to ß-lactam antibiotics, hydrolyzes the ß-lactam ring, rendering the ß-lactam inactive (Wilke et al., 2005). Methicillin resistance requires the presence of the chromosomally localized *mecA* gene (Chambers, 1997; Wilke et al., 2005). The *mecA* gene is responsible for synthesis of penicillin-binding protein 2a (PBP2a), an alternative transpeptidase with reduced affinity for ß-lactam antibiotics (Ghuysen, 1994; Hartman and Tomasz, 1984). Thus, resistance to methicillin confers resistance to all ß-lactam agents. PBP2a also differs from other PBPs in that its active site blocks binding of all ß-lactams but allows the transpeptidation reaction to proceed (Lim and Strynadka, 2002). Additional series of genes, the *fem* genes (factor essential for resistance to methicillin resistance) play a role in cross-linking peptidoglycan strands and also contribute to the heterogeneity of expression of methicillin resistance (Berger-Bächi, 1994).

Staphylococcal resistance to vancomycin in clinical isolates was first reported in a strain of *S. haemolyticus* (Schwalbe et al., 1987). Currently two forms of *S. aureus* resistance to vancomycin have been identified (Walsh and Howe, 2002). The reduced susceptibility to vancomycin appears to result from changes in peptidoglycan biosynthesis. The vancomycin intermediate-resistant *S. aureus* (VISA) strains are notable for the additional quantities of synthesized peptidoglycan that can result in irregularly shaped, thickened cell walls. There is also decreased cross-linking of peptidoglycan strands, which leads to the exposure of more D-Ala-D-Ala residues (Hanaki et al., 1998a, 1998b). The altered cross-linking results from reduced amounts of L-glutamine that are available for amidation of D-glutamate in the pentapeptide bridge (Walsh and Howe, 2002). The second form of vancomycin resistance has resulted from the probable conjugal transfer of the *vanA* operon from a vancomycin-resistant *Enterococcus faecalis* (Showsh et al., 2001). Resistance in these strains is caused by alteration of the terminal amino acids D-Ala-D-Ala to D-Ala-D-Lac. Synthesis of D-Ala-D-Lac occurs only with exposure to low concentrations of vancomycin.

Fluoroquinolones were initially introduced for the treatment of Gram-negative bacterial infections in the 1980s. Because of their Gram-positive bacterial spectrum, they have also been used to treat bacterial infections caused by pneumococci and staphylococci. Quinolone resistance among *S. aureus* emerged quickly, more

prominently among the methicillin-resistant strains. As a result, the ability to use fluoroquinolones as antistaphylococcal agents was dramatically reduced. Fluoroquinolone resistance develops as a result of spontaneous stepwise chromosomal mutations in the target of the antibiotic, topoisomerase IV or DNA gyrase, or by the induction of a multidrug efflux pump (Hooper, 2002).

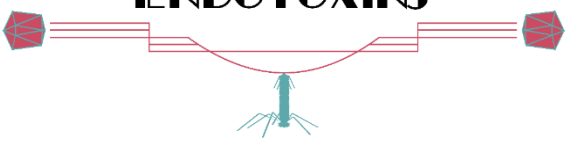
The confluence of high bacterial density, the likely preexistence of resistant subpopulations, and the sometimes limited quinolone concentrations achieved at sites of staphylococcal infections creates conditions that fosters selection of resistant mutants (Hooper, 2002). Amino acid changes in critical regions of the enzyme-DNA complex (quinolone resistance-determining region (QRDR)) reduce quinolone affinity for both of its targets. The ParC subunit (GrlA in *S. aureus*) of topoisomerase IV is the most common site of resistance mutations. Topoisomerase IV mutations are the most critical, since they are the primary drug targets in staphylococci (Ng et al., 1996). An additional mechanism of resistance in *S. aureus* is induction of the NorA multidrug resistance efflux pump. Increased expression of this pump in *S. aureus* can result in low-level quinolone resistance (Ng et al., 1994).

Table 3. 2: Mechanisms of Staphylococcus aureus resistance to antimicrobials. Adapted from Lowy (2003).

Antibiotic	Resistance gene	Gene product	Mechanism of resistance	Reference
ß-Lactams	*blaZ*	ß-Lactamase	Enzymatic hydrolysis of ß-lactam	Wilke et al. (2005)
	mecA	PBP2a	Reduced affinity for PBP	Chambers (1997); Hartmann and Krieg (1999); Hiramatsu (2001); Katayama et al. (2000); Wilke et al. (2005)
Glycopeptides		Altered peptidoglycan	Trapping of vancomycin in the cell wall by thickening the cell wall	Hanaki et al. (1998); Hanaki et al. (1998)
		D-Ala-D-Lac	Synthesis of dipeptide with reduced affinity for vancomycin	Levine (2006)
Quinolones	*parC*	ParC (or GrlA) component of topoisomerase IV	Mutations in the QRDR region, reduced affinity of enzyme–DNA complex for quinolones	Hooper (2002); Ng et al. (1996)
	gyrA or *gyrB*	GyrA or GyrB components of gyrase	Efflux pumps	

Chapter 4

Endotoxins



"If at first you don't succeed, try two more times so that your failure is statistically significant."

Unknown statistician

Introduction – Endotoxins

Both Gram-positive and Gram-negative bacteria possess a cytoplasmic membrane surrounding the cytosol, a double phospholipid layer which constitutes a physical semi-permeable barrier that regulates the flux of endogenous and exogenous substances in and out of the cell. Gram-negative bacteria possess an additional asymmetric outer membrane (OM) which surrounds a thin layer of peptidoglycan. The outer membrane is composed of an asymmetric phospholipid bilayer, whose inner leaflet is made of glycerophospholipids while the external leaflet is formed by lipopolysaccharides (LPS) (Silipo and Molinaro, 2010).

Lipopolysaccharides constitute a physical barrier protecting the bacterium from host defenses, mediate direct interactions with host cell receptors and antibiotics, and are potent signaling molecules which initiate some of the events leading to host tissue damage and much of the pathology associated with bacteremia (King et al., 2009; Moskowitz and Ernst, 2010; Raetz, 1990). They are heat-stable complex amphiphilic macromolecules indispensable for the bacterial growth, viability and for the correct assembly of the outer membrane (Alexander and Rietschel, 2001; Raetz and Whitfield, 2002; Raetz et al., 2007). Lipopolysaccharides typically consist of a hydrophobic domain known as lipid A (or endotoxin), a non-repeating core oligosaccharide, and a distal polysaccharide (or O-antigen) (Raetz and Whitfield, 2002). The biosynthetic pathway and export mechanism of LPS has been well characterized in *E. coli* and is shared by most Gram-negative bacteria, but the exact structures of LPS differ in different bacteria (Wang and Quinn, 2010). Lipopolysaccharides can bind to the PRR TLR4 (Akira et al., 2006; Poltorak et al., 1998).

Biosynthesis

Lipopolysaccharides can cause symptoms such as septic shock, multiple organ dysfunction and failure. Understanding the biochemistry of LPS modifications and their impact on pathogenesis could lead to novel treatment options for these diseases as well as lead to strategies for the removal of LPS from different sample types (Epstein and Parrillo, 1993; Petsch and Anspach, 2000).

The biosynthesis of LPS is initiated from a small molecule, UDP-*N*-acetylglucosamine (UDP-GlcNAc), which is also one of the two major building blocks of the peptidoglycan layer. Multiple enzymes sequentially function to convert UDP-GlcNAc into core-lipid A, and to synthesize LPS. Among the three parts of LPS, the structure of lipid A is more widely conserved in different bacteria than that of core sugars or O-antigen and so are the enzymes involved in the biosynthesis of lipid A. The first stage of LPS biosynthesis is to synthesize Kdo_2-lipid A (Figure 4. 1; Doerrler, 2006; Raetz and Whitfield, 2002).

The core oligosaccharides are sequentially assembled on lipid A at the cytoplasmic surface of the inner membrane in a process that involves a number of membrane-

associated glycosyltransferases, using nucleotide sugars as donors. The biosynthesis of core oligosaccharides is rapid and efficient, suggesting that the glycosyltransferases function as a coordinated complex. Core oligosaccharides can be divided into two structurally distinct regions: the inner core, which connects to lipid A, and the outer core that connects to the O-antigen. The inner core oligosaccharides typically contain residues of Kdo and L-glycero-D-manno-heptose (Hep). The Kdo residue is the most conserved component found in the core region of LPS. The outer core oligosaccharides show more structural diversity than those of the inner core. The sugars found in the outer core oligosaccharides are Kdo, Hep, D-glucose and D-galactose (Wang and Quinn, 2010).

Similar to the core oligosaccharides, O-antigen is synthesized at the cytoplasmic surface of the inner membrane. Using the sugar nucleotides as donors, the units of O-antigen are assembled by glycosyltransferase enzymes on the membrane-bound carrier, undecaprenyl phosphate which is also used for synthesis of peptidoglycan and capsular polysaccharides (Raetz and Whitfield, 2002). The O-antigens of LPS exhibit considerable diversity. The connection of units in O-antigen may be linear or branched. The O-antigen repeats can be homopolymers or heteropolymers (Wang and Quinn, 2010).

Nascent LPS molecules are synthesized in the periplasm and shuttled to the inner surface of the outer membrane by proteins LptA, LptB, LptC, LptF and LptG. The ABC transporter LptBFG, functioning with LptC and LptA, translocates LPS to the inner leaflet of the outer membrane (Sperandeo et al., 2007, 2008). Thereafter, the protein complex LptD/LptE assembles LPS into the outer surface of the outer membrane (Ruiz et al., 2008; Sperandeo et al., 2007, 2008).

After synthetization, the LPS structure can be modified. The most conserved part of lipid A is its backbone, the disaccharide of glucosamine, but the groups connecting to this backbone can be modified (Table 4. 2). The modification of LPS cannot only occur in the hydrophobic acyl chain (lipid A) but also in the hydrophilic polysaccharide (O-antigen). Modifications of LPS usually occur at the periplasmic face of the inner membrane or in the outer membrane. These structural modifications of LPS might help bacteria to resist the cationic antimicrobial peptides (CAMPS) released by the host immune system, or evade recognition by the innate immune receptor TLR4 (Wang and Quinn, 2010).

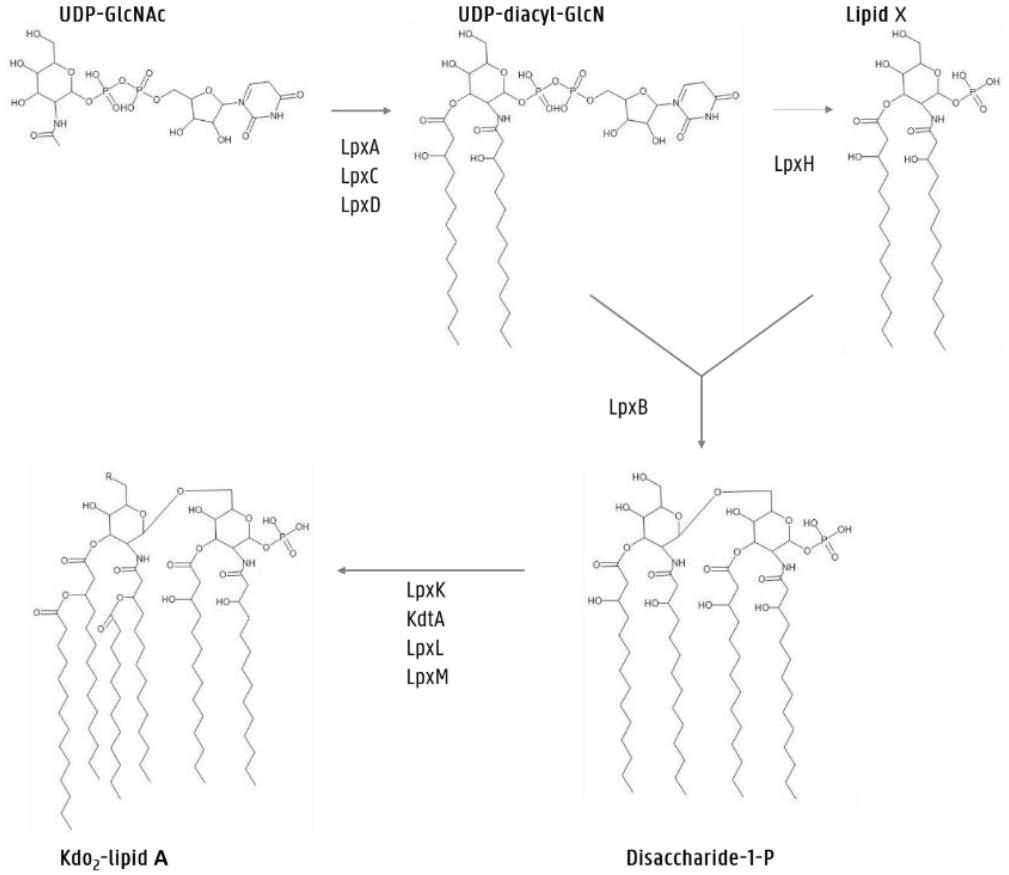


Figure 4. 1: **Structure and biosynthetic pathway of Kdo_2-lipid A in *Escherichia coli*.** This involves nine enzymes (Table 4. 1), takes place in the cytoplasm at first and ends up on the cytoplasmic surface of the inner membrane. The first three reactions of the Kdo_2-lipid A biosynthesis are catalyzed by soluble enzymes LpxA, LpxC and LpxD, resulting in the addition of two 3-OH fatty chains to the 2- and 3- positions of the UDP-GLCNAc to form UDP-diacyl-GlcN. Both LpxA and LpxD are acyltransferases and their active forms are homotrimers. LpxC is a Zn2+-dependent deacetylase which has no sequence homology with other deacetylases (Barb et al., 2007). UDP-diacyl-GlcN is subsequently hydrolyzed by LpxH to form lipid X, which is further condensed with its precursor UDP-diacyl-GlcN by LpxB to form disaccharide-1-P (Babinski et al., 2002a, 2002b, Crowell et al., 1986, 1987; Metzger IV and Raetz, 2009). In the final steps, LpxK phosphorylates the 4'-position of the disaccharide-1-P to form lipid IV A (Garrett et al., 1998). KdtA incorporates two 3-deoxy-D-manno-octulosonic acid (Kdo, depicted as an R-group) residues at the 6'-position of the lipid IV A, using a sugar nucleotide CMP-Kdo as the donor (not depicted in the figure). The resulting Kdo_2-lipid IV A undergoes further reactions catalyzed by LpxL and LpxM to form Kdo_2-lipid A. LpxL adds a secondary lauroyl residue and LpxM a myristoyl residue to the distal glucosamine unit. Figure adapted from *Wang and Quinn (2010)*.

Table 4. 1: **Information on nine enzymes required for the biosynthesis of Kdo_2-lipid A in *Escherichia coli.*** Adapted from Wang and Quinn (2010).

Enzyme	Function	Substrate	Donor	Reference
LpxA	Acyltransferase	UDP-GlcNac	R-3-hydroxymyristoyl ACP	Williams and Raetz (2007)
LpxC	Deacetylase	UDP-3-O-(acyl) GlcNac	None	Barb *et al.* (2007)
LpxD	Acyltransferase	UDP-3-O-(acyl)-GlcN	R-3-hydroxymyristoyl ACP	Buetow *et al.* (2007)
LpxH	Pyrophosphatase	UDP-2,3-diacyl-GlcN	None	Babinski *et al.* (2002)
LpxB	Disaccharide synthase	UDP-2,3-diacyl-GlcN; Lipid X	None	Crowell *et al.* (1987; 1986)
LpxK	4'-kinase	Disaccharide 1-phosphate	None	Garrett *et al.* (1998; 1997)
KdtA	Kdo transferase	Lipid IV_A	CMP-Kdo	Brozek *et al.* (1989)
LpxL	Acyltransferase	Kdo_2-lipid IV_A	Lauroyl ACP	Brozek and Raetz (1990)
LpxM	Acyltransferase	Kdo_2-penta-lipid A	Myristoyl ACP	Brozek and Raetz (1990)

Membrane proteins PgaP, PagL, LpxR and LpxO have been reported to modify the fatty acyl chain region of LPS. PagP is a palmitoyl transferase which locates in the outer membrane and transfers a palmitate from glycerophospholipids to the ß2-position of lipid A resulting in a hepta-acylated structure (Ahn et al., 2004). PagL is a lipase that removes the 3-O-linked acyl chain of lipid A but plays no role in antimicrobial peptide resistance (Kawasaki et al., 2004). The negative charge of Lipid A allows the binding of positively charged cAMPs produced by the immune system. To evade the attack by the immune system some bacterial pathogens have evolved less negatively charged variations of lipid A by removing or decorating the phosphate groups at the 1- and 4'-positions. The decoration includes the addition of amine-containing residues such as α-L-Ara4N and phosphoethanolamine. The modifications result in resistance to cAMPs and are controlled by the PmrA-PmrB two-components system (Wang and Quinn, 2010; Wang et al., 2004). Another strategy that bacteria employ to decrease the surface negative charge is the addition of amino groups at 1- and 4'-phosphate of lipid A. EptA is necessary for addition of phosphoethanolamine to the 1-phosphate of lipid A (Lee et al., 2004).

Table 4. 2: Enzymes involved in the structural modification of LPS in Gram-negative bacteria. Adapted from Wang and Quinn (2010).

Enzyme	Function	Reference
LpxE	Remove the phosphate group from the 1-position of lipid A	Wang *et al*. (2004)
LpxF	Remove the phosphate group from the 4'-position of lipid A	Wang *et al*. (2006)
LpxO	Add an OH group to the $\alpha_{\beta 3'}$-position of lipid A	Gibbons *et al*. (2008; 2000)
Arnt	Transfer the L-Ara4N unit to lipid A	Trent *et al*. (2001)
LpxR	Remove the 3'-acyloxyacyl moiety of lipid A	Reynolds *et al*. (2006)
PagL	Remove the 3-O-linked acyl chain of lipid A	Rutten *et al*. (2006)
PagP	Transfer a palmitate to the $ß_2$-position of lipid A	Bishop (2008); Hwang *et al*. (2004)
LpxXL	Add a very long fatty acid chain to the $ß_{2'}$-position of lipid A	Haag *et al*. (2009)
LpxT	Transfer a phosphate group to the 1-phosphate of lipid A	Touzé *et al*. (2008)
LpxQ	Oxidize the proximal glucosamine of lipid A to form an aminogluconate unit	Que-Gewirth *et al*. (2003)
LmtA	Catalyze the methylation of 1-phosphate of lipid A	Boon Hinckley *et al*. (2005)
RgtA	Add a GalA moiety to the distal unit of Kdo	Kanjilal-Kolar *et al*. (2006)
RgtB	Add a GalA moiety to the distal unit of Kdo	Kanjilal-Kolar *et al*. (2006)
RgtC	Add a GalA moiety to the mannose residue of the core oligosaccharide of LPS	Kanjilal-Kolar *et al*. (2006)
EptA	Add a phosphoethanolamine to 1-position of lipid A	Lee *et al*. (2004)
EptB	Add a pEtN moiety to the distal unit of Kdo	Reynolds *et al*. (2005)

Immunological response towards endotoxins

Lipopolysaccharides have been shown to be among the most powerful classes of immunostimulators known to physiologically function as specific indicators for infection by Gram-negative bacteria of diverse eukaryotes ranging from man to insect. In general, the lipid A region represents the primary immunoreactive center of LPS due to the specific and often highly sensitive recognition of this bacterial lipid structure by numerous cellular and humoral components of innate immunity (Hoffmann et al., 1999; Janeway and Medzhitov, 2002; Mackay et al., 2000). Lipopolysaccharides commonly induce a broad spectrum of biological effects in

various eukaryotic organisms. The primary target cells of LPS in mammalian species are the professional phagocytes of innate or natural immunity, *i.e.* peripheral monocytes, tissue macrophages and neutrophils, which constitutively express the membrane-bound form of the CD14 antigen as well as TLR4 (Haziot et al., 1988; Kitchens, 2000; Muzio et al., 1998, 2000; Zhang et al., 1999a).

Lipopolysaccharide stimulation of mammalian cells occurs through a series of interactions with several proteins including the LPS binding protein (LBP), CD14, MD-2 and TLR4 (Figure 4. 1)(Gioannini and Weiss, 2007; Miyake, 2007). LBP is a soluble shuttle protein which directly binds to LPS and facilitates the association between LPS and CD14 (Tobias et al., 1986; Wright et al., 1989). CD14 is a glycosylphosphatidylinositol-anchored protein, which also exists in a soluble form. CD14 facilitates the transfer of LPS to the TLR4/MD-2 receptor complex and modulates LPS recognition (Wright et al., 1990). MD-2 is a soluble protein that non-covalently associates with TLR4 but that can directly form a complex with LPS in the absence of TLR4 (Gioannini and Weiss, 2007; Nagai et al., 2002; Shimazu et al., 1999).

Upon LPS recognition, TLR4 undergoes oligomerization and recruits its downstream adaptors through interactions with the TIR domains. TIR domains contain three highly conserved regions, which mediate protein-protein interactions between the TLRs and signal transduction adaptor proteins. The TIR domain of TLR4 is critical for signal transduction, because a single point mutation in the TIR domain can abolish the response to LPS (Poltorak et al., 1998). In total, there are five TIR domain-containing adaptor proteins: MyD88, TIRAP, TRIF, TRAM and SARM (O'Neill and Bowie, 2007). TLR4 signaling has been divided into MyD88-dependent and MyD88-independent (TRIF-dependent) pathways (Figure 4. 2A and B). The MyD88-dependent pathway was shown to be responsible for pro-inflammatory cytokine expression, while the MyD88-independent pathway mediates the induction of type I interferons and interferon-inducible genes (Lu et al., 2008).

The MyD88-dependent pathway

In addition to the TIR domain, MyD88 also contains a death domain (DD), which can recruit other DD-containing molecules through homotypic interactions. Upon LPS stimulation, MyD88 recruits and activates a DD-containing kinase, IL1 receptor associated kinase-4 (IRAK-4) (Suzuki et al., 2002). IRAK-4 also plays a role in the mRNA stability of certain cytokines and chemokines, such as TNFα (Kim et al., 2007).

Another adaptor protein TRAF6 (TNF receptor-associated factor 6), is critical for the MyD88-dependent pathway downstream of IRAK4 and IRAK1 (Figure 4. 2A). TRAF6 forms a complex with UBC13 (ubiquitin-conjugating enzyme 13) and UEV1A (ubiquitin-conjugating enzyme E2 variant 1 isoform A), and activates TAK1 (transforming growth factor-ß-activated kinase) (Gohda et al., 2004; Lomaga et al.,

1999). TAK1 then activates downstream IKK (IκB kinase) and MAPK (mitogen-activated protein kinase) pathways (Sato et al., 2005). IKKα, IKKß and IKKγ form a complex and phosphorylate IκB proteins. This phosphorylation leads to the degradation of IκB proteins and the subsequent translocation of NF-κB, which controls the expression of pro-inflammatory cytokines, in addition to other immune related genes (Lu et al., 2008).

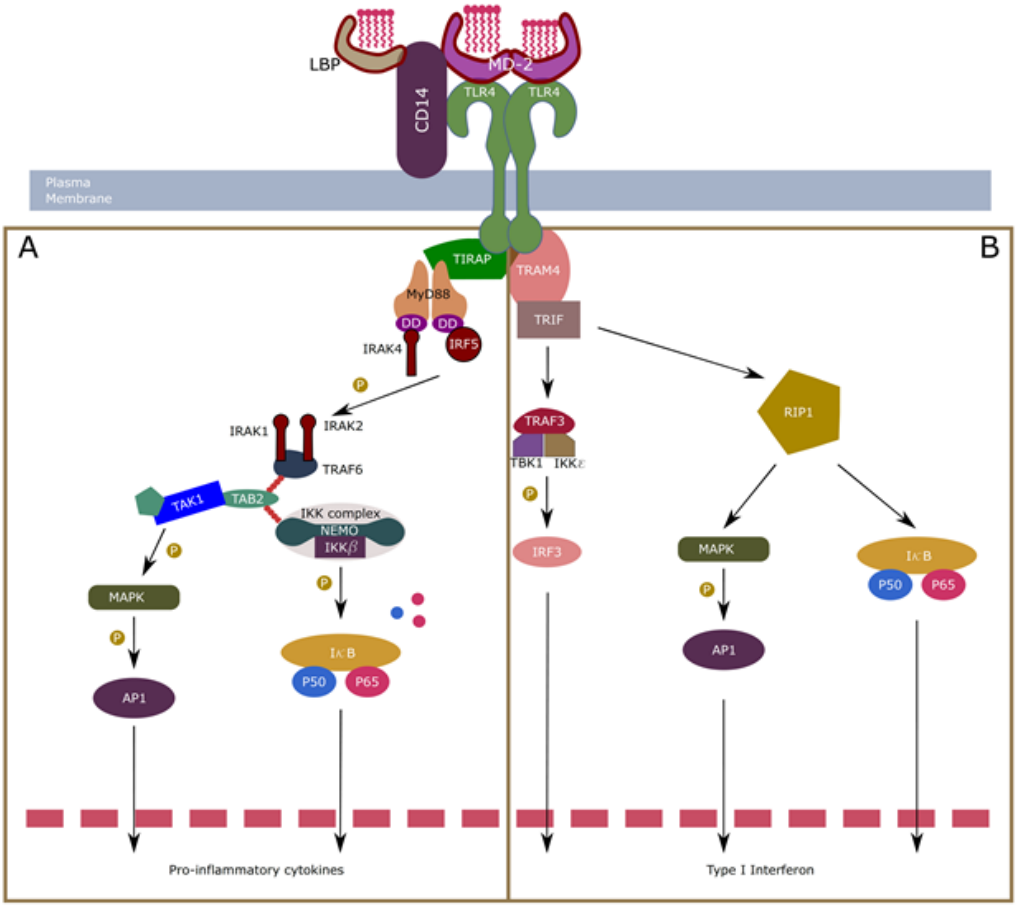


Figure 4. 2: Overview of LPS/TLR4 signaling. LPS recognition is facilitated by LBP and CD14, and is mediated by TLR4/MD-2 receptor complex. LPS/TLR4 signaling can be separated into (**A**) MyD88-dependent and (**B**) MyD88-independent pathways. **A| MyD88-dependent pathway**. MyD88 activates IRAKs/TRAF6 as well as the transcription factors NF-κB and AP-1. These transcription factors induce expression of pro-inflammatory cytokines. **B| MyD88-independent pathway**. TRIF signals the induction of Type I interferons by recruiting TRAF3 and RIP1 to activate transcription factor IRF3, as well as NF-κB and AP-1. Figure adapted from Lu *et al.* (2008).

The MyD88-independent pathway

TRIF is an important TIR-containing adaptor protein that mediated MyD88-independent signaling (Figure 4. 2B). The C-terminal region of TRIF, which contains a Rip homotypic interaction motif (RHIM), mediates the interaction with RIP1 (receptor-interacting protein 1). As a serine/threonine kinase, RIP1 was initially identified as an important component of TNFα-mediated NF-κB activation (Meylan et al., 2004). TRIF recruits TRAF3 to activate IRF3. TRAF3 can associate with TANK (TRAF family member-associated NF-κB activator), TBK1 (TANK binding kinase 1) and IKKi to mediate downstream signaling (Guo and Cheng, 2007; Oganesyan et al., 2006). TBK1 and IKKi are important for the dimerization and translocation of IRF3 (Fitzgerald et al., 2003; Hemmi et al., 2004). IRF3, together with NF-κB, activates the transcription of target genes, such as type I interferons (Honda and Taniguchi, 2006; Moynagh, 2005). The induction of type I interferons and interferon-inducible genes are important for anti-viral and anti-bacterial responses (Bowie and Haga, 2005; Perry et al., 2005).

Endotoxin removal

Endotoxins liberated by Gram-negative bacteria are frequent contaminants of protein solutions derived from bioprocesses. Due to their toxicity *in vivo* and *in vitro*, their removal is essential for safe parenteral administration (Petsch and Anspach, 2000). The threshold level of endotoxins for intravenous applications is set to five endotoxin units (EU) per kg body weight per hour by all pharmacopoeias (Council of Europe., 1997). The term EU describes the biological activity of an endotoxin. As a rule of thumb, one EU corresponds to 100 pg of endotoxins; the amount present in 10^5 bacteria. Meeting this threshold level, knowing that a single *E. coli* contains about two million LPS molecules per cell, has always been a challenge in biological research and pharmaceutical industry (Berthold and Walter, 1994; de Oliveira Magalhães et al., 2007).

Common purification strategies that include several chromatographic steps, such as ion exchange, hydrophobic interaction chromatography and gel filtration, may provide sufficient endotoxin clearance. Generally, high endotoxin concentrations can be reduced to about 100 EU/ml without special treatment (Petsch and Anspach, 2000). The most secure way to avoid any microbial contamination and with it the release of endotoxins is absolute sterility during the production and downstream processes. Yet, if a decontamination method is used, it must ensure a high recovery of the target product. At present, three endotoxin removal strategies are available for the removal of LPS from protein samples, *i.e.*, ultrafiltration, two-phase extraction, and adsorption (such as anion-exchange chromatography and affinity adsorption) (Petsch and Anspach, 2000).

Ultrafiltration. Gel filtration chromatography reveals that more than 80 % of the endotoxin activity of a protein-free solution elutes as aggregates with the void volume (Morrison and Leive, 1975). Ultrafiltration can also be used to remove

endotoxins from product solutions if the products are low of molecular weight (Petsch and Anspach, 2000).

Two-phase extraction. Through addition of detergents, an improvement of chromatographic protocols is possible (Karplus et al., 1987). Above the critical micellar concentration (CMC) of detergents, endotoxins are accommodated in the micellar structure by non-polar interactions of the alkyl chains of lipid A and the detergent and are consequently separated from the water phase (Brandenburg and Wiese, 2004; Israelachvili, 2011). Detergents of the Triton series show a miscibility gap in aqueous solutions. Above a critical temperature, the so-called cloud point, micelles aggregate to droplets with very low water content, as such forming a new phase. Endotoxins remain in the detergent-rich phase. Through centrifugation or further increase in temperature the two phases separate with the detergent-rich phase being the bottom phase (Aida and Pabst, 1990; Bordier, 1981). If necessary, this process is repeated until the remaining endotoxin concentration is below the threshold limit. The cloud point of Triton X-114 is at 22 °C, which is advantageous when purifying proteins. It requires mixing of the endotoxin-containing protein solution in the cold (usually at 4 °C) and allows separation of the two phases at T > 22 °C. In contrast, the cloud point for Triton X-100 is at 75°C, which is not acceptable for most proteins (Petsch and Anspach, 2000).

Adsorption techniques. Most frequently, adsorption techniques are employed for the removal of endotoxins from protein solutions. In principle, non-selective adsorption on activated carbon or other adsorber materials is possible (Nagaki et al., 1991). Since endotoxins are negatively charged, anion exchangers are employed for their adsorption from protein-free solutions, such as DEAE chromatographic matrices or DEAE membranes or matrices functionalized with quaternary amino groups (Gerba and Hou, 1985; Hou and Zaniewski, 1990a, 1990b; Neidhardt et al., 1992). Clearance factors of more than five orders of magnitude can be obtained. According to the concept of affinity interactions, clearance by an endotoxin-selective affinity sorbent should be possible and should guarantee a protein recovery of almost 100% (Petsch and Anspach, 2000).

Endotoxin detection

Most types of lipid A molecules are detected at picomolar levels by an ancient receptor of the innate immune system present on macrophages and endothelial cells of animals (Raetz and Whitfield, 2002). Indeed, cells which are highly sensitive to LPS or lipid A have been detected in the hemolymph system of the American horseshoe crab *Limulus polyphemus*, and its Asian variant *Tvoluit tridendatus*, which have existed more than 400 million years.

The horseshoe crab hemolymph contains mainly one type of blood cells called amoebocytes, which are extremely sensitive to LPS. During a Gram-negative infection, the amoebocytes release granular components into the plasma to participate in self-defense via blood coagulation, which incapacitates the invading

microbe (Iwanaga and Lee, 2005). Lipopolysaccharides from Gram-negative bacteria induces the amoebocytes to degranulate, thus initiating the blood coagulation cascade (Armstrong and Rickles, 1982). This cascade is based on three serine proteases zymogens, Factor C, Factor B, proclotting enzyme and one clottable protein, coagulogen (Figure 4. 3) (Muta and Iwanaga, 1996). Factor C, at the first step of the coagulation pathway, is sensitive to LPS and is a unique LPS-binding protein found only in the horseshoe crab. In the presence of LPS, the Factor C serine protease zymogen is automatically activated to an active form, which activates the proenzyme Factor B. This in turn activates proclotting to active clotting enzyme. Clotting enzyme then converts coagulogen into a coagulin clot, which traps the invading bacteria (Ding and Ho, 2010).

This evolutionary ancient system, originally described by Levin and Bang in 1964 (Levin and Bang, 1964), provided the basis for the development of the *limulus* amoebocyte lysate (LAL) assay, which today is a standard procedure for the detection of LPS or free lipid A in diverse settings (Hurley, 1995; Levin, 1988; Levin and Bang, 1964).

After two to three decades of conventional pyrogen testing using the blood extract of the horseshoe crab, and the problems associated with LAL, Factor C was recombinantly expressed (Rotundicauda, 1998). This yielded an enzymatically active recombinant Factor C (rFC) that is activated by trace levels of LPS, with a remarkable sensitivity of 0.001 EU/ml. Furthermore, being capable of binding both free and bound LPS/lipid A with high affinity, the rFC has other potential applications, such as the removal of LPS from contaminated samples (Ding and Ho, 1999). rFC is a proenzyme until it encounters trace levels of endotoxin where it unequivocally exhibits full enzymatic activity, hence, acting as a very sensitive and specific biosensor for endotoxin. The resulting activated rFC acts as a catalyst to hydrolyse a synthetic substrate to form a quantifiable, fluorimetric, product, which measures the level of endotoxins. A comparison of rFC with commercial LAL, under the same assay conditions, showed that rFC has a lower background reading and a more sensitive response to endotoxin (Ding and Ho, 2001).

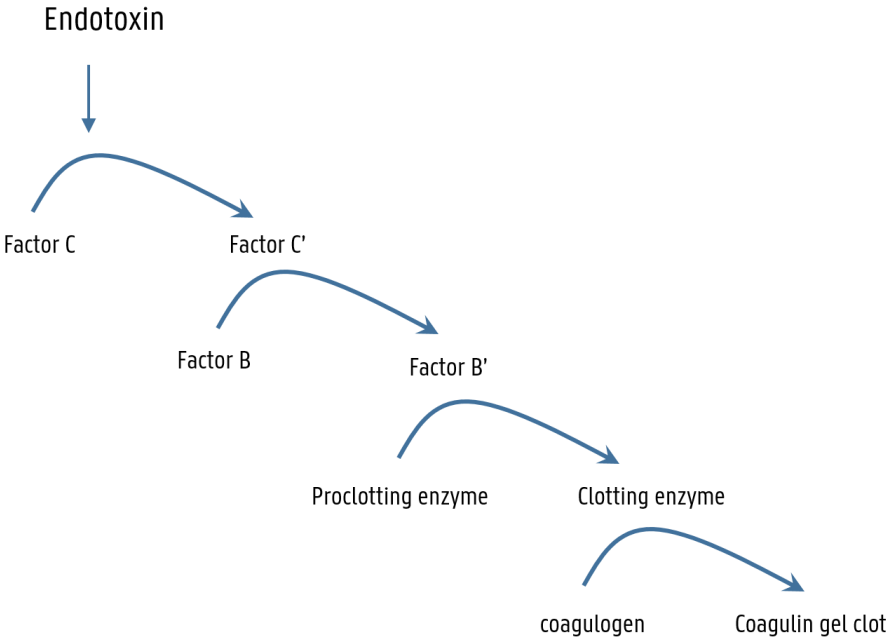
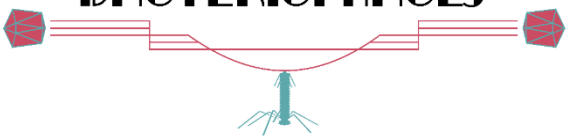


Figure 4. 3: The coagulation cascade in the horseshoe crab amoebocyte lysate. In the presence of the endotoxin LPS, the Factor C serine protease zymogen is autocatalytically activated to an active form, Factor C', which activates the proenzyme Factor B into Factor B'. This in turn activates the proclotting to the active clotting enzyme. The clotting enzyme then converts coagulogen into a coagulin gel clot, which traps the invading bacteria. Figure adapted from Ding and Ho (2010).

The disadvantage of the LAL assay, even when rFC is used, is that the a part of the sample is used as a whole. This means that certain rFC-inhibiting or rFC-activating components may be present, which could lead to either a false negative or false positive result. For this reason an alternative method has been developed, called EndoLISA. This method uses phage-derived receptor proteins, exhibiting high affinity and specificity for the conserved core region of LPS, that are immobilized on a microplate. This enables, after binding of the sample LPS to the microplate, to wash the original sample matrix off, thereby eliminating potentially interfering components. Subsequently the LPS is detected by factor C (Grallert et al., 2011).

Chapter 5

Bacteriophages



"It might be possible that a civilization more advanced than ours prepares an artificial or artificially modified phage (or bacterial) DNA which is capable of proliferating actively under suitable conditions and at the same time carries an intelligent message encoded in its base sequence."

Yokoo and Oshima, 1979

Introduction – Viruses of microbes

Viruses are a group of biological entities with a genome consisting of either DNA or RNA and encapsulated in a protein coat (capsid), and sometimes even with a lipid membrane. The species concept has been applied to viruses and a viral species is defined as a polythetic class of viruses that constitutes a replicating lineage and occupies a particular ecological niche (van Regenmortel, 1992).

Bacterial viruses were discovered twice, by Twort in 1915 and by d'Herelle in 1917 (Summers, 2011, 2016). There are constant debates on whether the discovery of bacterial viruses should be attributed to Félix d'Herelle or to Frederick William Twort (Duckworth, 1976). d'Herelle coined the name 'bacteriophages' for these infectious agents lysing bacteria, which literally means 'eaters of bacteria'. As it often happens in science, it is not enough to discover something new. It is equally important to see the possible applications of a new discovery. Félix d'Herelle clearly recognized the viral nature of his agent and devoted the rest of his scientific carreer to it. He also pioneered several principle lines of bacteriophage research by introducing phage treatment of bacterial infections, and by hinting that bacteriophages are suitable for research on the nature of the gene (Summers, 2016). It is therefore that he can be seen as the true 'phage father'. Research on (bacterio)phages played a central role in deciphering molecular principles of life such as the finding that DNA is the hereditary molecule and led to the development of an entirely new science, molecular biology (Duckworth, 1987). Right after their discovery, phages were also used in an early form of biotechnology to fight bacterial pathogens (Levin and Bull, 1996). In Western Europe and the United states, phage therapy was abandoned due to ambiguous results and the discovery of antibiotics (Sulakvelidze, 2001).

Bacteriophages occur everywhere in the biosphere and have colonised even such forbidding habitats as volcanic hotsprings. Their main habitats are the oceans and topsoils (Ackermann, 2011). Phages have double-stranded (ds) or single-stranded (ss)DNA or RNA. Besides tailed phages, also cubic, spindle, lemon-shaped, filamentous, pleomorphic and even 'hairy' phages have been described (Ackermann and DuBow, 1987; Børsheim, 1993; Bradley, 1967; Buttimer et al., 2017; Demuth et al., 1993; Frank and Moebus, 1987; Proctor, 1997).

Tailed phages constitute the order *Caudovirales* with three families, characterized by contractile (*Myoviridae*), long and noncontractile (*Siphoviridae*), or short tails (*Podoviridae*). Caudovirales represent over 96 % of known phages (Ackermann, 2011). A typical *Caudovirales* phage has a head and a tail, hold together by a connector.

The phage life cycle

Several steps during the life cycle of a prokaryotic virus can be distinguished that are common to all viruses: adsorption, separation of nucleic acids from protein coat,

expression and replication of the nucleic acids, virion assembly, release, and transmission (Weinbauer, 2004). Phage adsorption occurs in two steps. The first step of adsorption to a defined cell surface structure is reversible. During the second step, an irreversible binding between a phage structure (*e.g.* tail fibers) and the receptor on the host bacterium is accomplished. After adsorption, the cell wall is made penetrable, using tail associated lysins, and the nucleic acid is transported into the cell, whereas the capsid remains outside the cell. Following injection, the genetic material is either integrated into the host genome or stays in the cytoplasm. Phages can show several life cycles, *i.e.* lytic, lysogenic, chronic infection or pseudolysogenic (Figure 5. 1)(Ackermann and DuBow, 1987).

Lytic life cycle. In the lytic life cycle, the phage genome exists within the host but outside the host genome. Lytic or virulent phages repeat a cycle in which self-proliferation is synchronous with the destruction of bacteria (*i.e.* the lytic cycle or the virulent infection; Matsuzaki et al., 2005). In this stage, gene expression, genome replication and morphogenesis occurs, *i.e.* the formation of the genomes and the capsids (and tails) and the packing of the genomes in the capsids (Ackermann, 1998).

Lysogenic life cycle. In the lysogenic cycle, the genome of the (temperate or lysogenic) phage is integrated in the chromosome of the host (prophage) and replicates along with the host (now called a lysogen), until the lytic cycle is induced (Ackermann, 1998; Weinbauer, 2004).

Chronic infection. Chronically infecting phages produce progeny that are constantly released from the host cell by budding or extrusion without lysis of the host cell (Weinbauer, 2004).

Pseudolysogenic life cycle. Pseudolysogeny and carrier-state are widely used as synonyms. Pseudolysogeny can be defined as the stage of stalled development of a bacteriophage in a host cell without either multiplication of the phage genome (as in lytic development) or its replication synchronized with the cell cycle and stable maintenance in the cell line (as in lysogenization), which proceeds with no viral genome degradation, thus allowing the subsequent restart of virus development (Łoś and Węgrzyn, 2012). This phenomenon is usually caused by unfavorable growth conditions for the host cell (such as starvation) and is terminated with initiation of either true lysogenization or the lytic cycle when growth conditions improve (Paul and Jiang, 2001). Pseudolysogeny has been known for tens of years; however, its role has often been underestimated. Currently, it is being considered more often as an important aspect of phage-host interactions. Pseudolysogeny seems to play an important role in phage survival, as bacteria in a natural environment are often starved or their growth is very slow. This phenomenon can be an important aspect of phage-dependent bacterial mortality and may influence the virulence of some bacterial strains (Łoś and Węgrzyn, 2012).

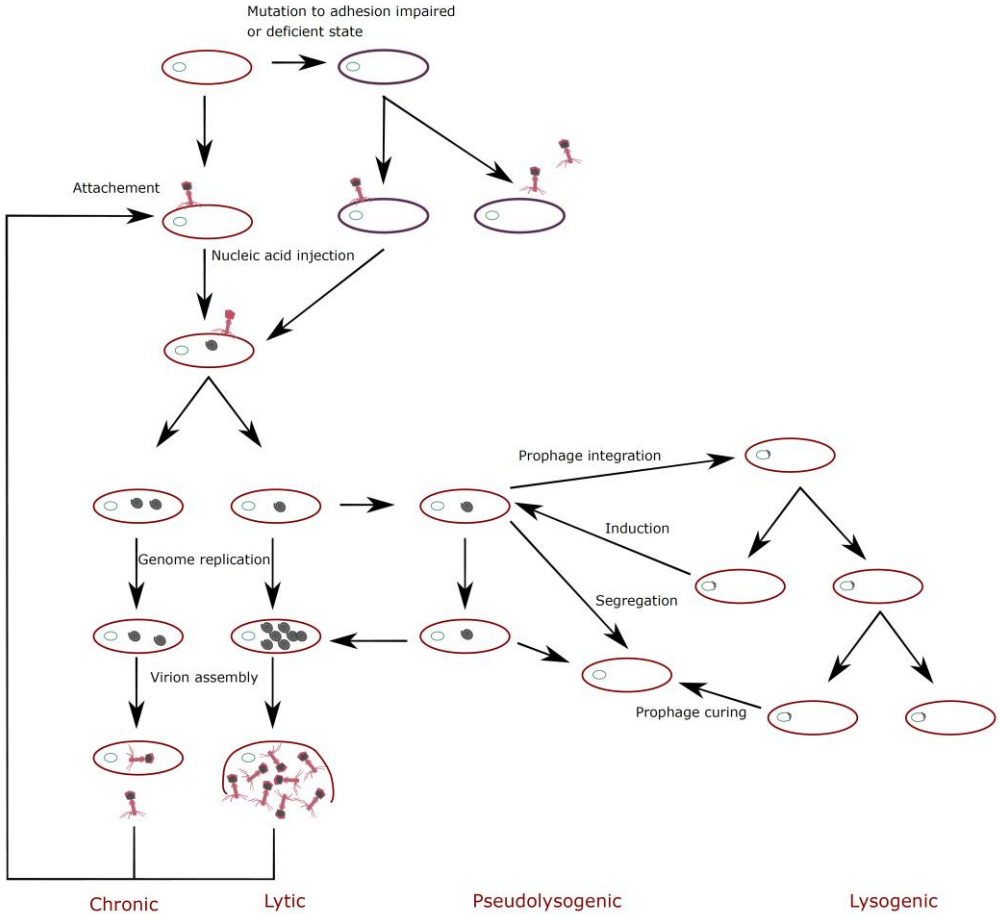


Figure 5. 1: Types of phage life cycles. Due to spontaneous mutations bacteria can become resistant to the phage infection, which the phage can overcome by slight adaptations in its tail fibers. After phage adsorption, phage DNA is injected into the bacterium (represented as a coiled molecule). This can become integrated into the host chromosome as a prophage in lysogeny. By the process of induction, the prophage is excised, and can go into the lytic phage replication. In pseudolysogeny, the host cell can mutate to an adhesion-impaired or deficient state, whereby collisions result in a low success rate of infection. Another type of pseudolysogeny, termed carrier state, can occur when the prophage does not integrate but is maintained as a plasmid. Both types of pseudolysogeny result in a high abundance of both phages and host cells simultaneously. When phages continuously produce progeny phages through budding or extrusion without lysis of the host cell, is a process called chronic infection. Adapted from Weinbauer (2004).

However, the carrier state is more strictly used for bacteria with a plasmid-like prophage. Mechanisms for establishing the carrier state include a reduced success rate of infection conferred by limited available receptors or by enzymatic loss of

receptors or superinfection immunity of a temperate phage (Barksdale and Arden, 1974).

Bacterial phage resistance mechanisms

Bacterial cells and their viral predators (phages) are locked in a constant battle. In order to proliferate in phage-rich environments, bacteria have evolved an impressive arsenal of defense mechanisms, and in response, phages have evolved counter-strategies to evade these antiviral systems (Samson et al., 2013). Bacterial antiphage systems include the inhibition of phage attachment to cell surface receptors, cleavage of the invading phage genome and even the induction of an altruistic cell suicide to abort phage infection. However, despite this arsenal, a large proportion of bacteria succumb to phage infection. Owing to their genomic plasticity and rapid multiplication rates, phages have evolved equally diversified strategies to thrive in apparently well-protected bacterial cells (Labrie et al., 2010).

Preventing phage adsorption

Adsorption of phages to host receptors is the initial step of infection and, perhaps, one of the most intricate events, as phages must recognize a particular host-specific cell component.

Blocking phage receptors. To efficiently attach to the surface of its bacterial host, a phage targets cell surface receptors. Given that adsorption is often intricately coupled to the injection of phage DNA, and that both of these interdependent steps must be achieved to enable intracellular phage replication, the specific interaction between the phage receptor-binding protein and its bacterial cell surface receptor is one of the primary parameters defining phage infection kinetics (Bertin et al., 2011; Moldovan et al., 2007). To limit phage propagation, bacteria can adapt the structure of their cell surface receptors or their three-dimensional conformation. *S. aureus,* for example, produces a cell-wall-anchored virulence factor, immunoglobulin G-binding protein A, which binds to the Fc fragment of immunoglobulin G (Foster, 2005). It has been shown that phage adsorption improves when bacteria produce less protein A, indicating that this protein masks the phage receptor (Nordström and Forsgren, 1974). On the other hand, tailed phages can evolve to modify their receptor-binding proteins to acquire novel receptor tropism (Samson et al., 2013). Furthermore, some lysogenic phages, *e.g. P. aeruginosa* phage D3, is able to alter the LPS structure of the *P. aeruginosa* infected bacteria. This protects the lysogen from being infected and lysed by the same phage (Kropinski, 2000).

Production of extracellular matrix. The production of structured extracellular polymers can promote bacterial survival in various ecological niches by protecting the bacteria against harsh environmental conditions and, in some cases, providing a physical barrier between phages and their receptors. Some phages have also evolved to specifically recognize these extracellular polymers and even to degrade them (Stummeyer et al., 2006; Sutherland, 1995, 1999; Sutherland et al., 2004).

Polysaccharide-degrading enzymes can be classified into two groups: the lyases and the hydrolases. The lyases cleave the linkage between the monosaccharides and the C4 of uronic acid and introduce a double bond between the C4 and C5 of uronic acid (Sutherland, 1999). The hydrolases break the glycosyl-oxygen bond in the glycoside linkage (Sutherland, 1995). These viral enzymes are found either bound to the phage structure (connected to the receptor-binding complex) or as free soluble enzymes from lysed bacterial cells (Sutherland, 1995).

Production of competitive inhibitors. Molecules that are naturally present in the bacterial environment can bind specifically to the phage receptors, rendering these receptors unavailable for phages (Labrie et al., 2010). For example, *E. coli* FhuA is an iron transporter, but also a port of entry for phage T1 and T5. The anti-microbial molecule microcin J25, produced under conditions of nutrient depletions and plays a role in the growth inhibition of phylogenetically related strains, also uses FhuA as a receptor and can outcompete phage T5 for binding to FhuA (Destoumieux-Garzón et al., 2005).

Preventing phage DNA entry

Superinfection exclusion (Sie) systems are proteins that block the entry of phage DNA into the host cells, thereby conferring immunity against specific phages. These proteins are predicted to be membrane anchored or associated with membrane components. The genes encoding these proteins are often found in prophages, suggesting that in many cases Sie systems are important for phage-phage competition rather than phage-host interactions.

Coliphage T4, a well-characterized virulent phage, has two Sie systems encoded by *imm* and *sp*. These systems cause rapid inhibition of DNA injection into cells, preventing subsequent infection by other T-even-like phages. Imm prevents the transfer of phage DNA into the bacterial cytoplasm by changing the conformation of the injection site. Imm has two non-conventional transmembrane domains and is predicted to be localized to the membrane, but Imm alone does not confer complete phage immunity and must be associated with another membrane protein to exert its function and achieve complete exclusion (Lu et al., 1993). The membrane protein Sp inhibits the activity of the T4 lysozyme, thereby preventing the degradation of peptidoglycan and the subsequent entry of phage DNA. The T4 lysozyme is found at the extremity of the tail and creates holes in the cell wall, facilitating the injection of phage DNA into the cell (Lu and Henning, 1994; Moak and Molineux, 2000).

Cutting phage nucleic acids

Restriction-modification systems. Many bacterial genera possess restriction-modification (R-M) systems. Their activities are due to several heterogeneous proteins that have been classified into at least four groups (type I – type IV). The principal function of the R-M system is thought to be protecting the cell against invading DNA, including viruses (Pingoud et al., 2005; Robinson et al., 2001).

When unmethylated phage DNA enters a cell harboring a R-M system, it will be either recognized by the restriction enzyme and rapidly degraded or, to a lesser extent, methylated by a bacterial methylase to avoid restriction, therefore leading to the initiation of the phage's lytic cycle (Krüger and Bickle, 1983).

The CRISPR-Cas system. The clustered regulatory interspaced short palindromic repeats (CRISPRs) and the CRISPR-associated (*cas*) genes provide bacteria and archaea with adaptive immunity against phages and plasmids. The mechanisms of action of CRISPR-Cas systems can be divided in three stages: acquisition, expression and maturation, and interference (Amitai and Sorek, 2016). The acquisition stage contains the recruitment of new spacers. In this stage the Cas proteins identify the target (phage) DNA and acquire a new spacer from their targets. This spacer sequence is integrated into the CRISPR array and forms the immunological memory, which reflects the chronology of past infections (van der Oost et al., 2014). The expression stage contains the transcription of the CRISPR array and subsequent processing of the precursor transcript into smaller CRISPR RNAs (crRNAs). The crRNA-directed cleavage of invading DNA by Cas nucleases forms the interference stage (Amitai and Sorek, 2016; van der Oost et al., 2014; Sampson et al., 2013).

Abortive infection systems

Bacteria carry a wide range of heterologous proteins that provide resistance through the abortion of phage infection. These abortive infection (Abi) systems also lead to the death of the infected cell, which is not the case for the antiphage systems described above. Typically, these Abi systems target a crucial step of phage multiplication such as replication, transcription or translation (Labrie et al., 2010). Abortive infection results in the destruction of the cell before phages can replicate. This suicidal sacrifice protects the cells of the same population from infection by phages (Forde and Fitzgerald, 1999; Weinbauer, 2004)

Phage therapy

With the rising prevalence of antibiotic-resistant bacteria and the serious concerns raised by the World Health Organization (WHO), new approaches to deal with bacterial infections have become an urgent need (Levy and Marshall, 2004). Phage therapy is the application of bacteria-specific viruses to combat bacterial infections (Summers, 2001).

The advantages of phage therapy over the use of chemical antibiotics can be framed in terms of the phage properties: *i.e.* the lytic activity of the phages, auto-dosing, low inherent toxicity, minimal disruption of normal microflora, narrower potential for inducing resistance (and if resistance against a specific phage is observed, new phages can be easily isolated), lack of cross-resistance with antibiotics, rapid discovery, formulation and application versatility and possibly biofilm clearance (Loc-Carrillo and Abedon, 2011). Auto–dosing phages are capable of increasing their

number during the bacterial-killing process and disappear after the target is cleared. The term auto dosing refers to the fact that phages themselves contribute to establishing the phage dose (Carlton, 1999; Chan and Abedon, 2012; Skurnik and Strauch, 2006). Finally, phages, like antibiotics, can be versatile in terms of formulation development, such as being combined with certain antibiotics (Alisky et al., 1998; Kutter et al., 2010). They are also versatile in application form, as liquids, creams, impregnated into solids, in addition to being suitable for most routes of administration (Carlton, 1999; Kutateladze and Adamia, 2010; Kutter et al., 2010). Different phages can be mixed as cocktails to broaden their properties, typically resulting in a collectively greater antibacterial spectrum of activity and possibly limiting the risk of resistance development(Goodridge, 2010; Merabishvili et al., 2009).

Phages as pharmaceuticals are protein-based, infectious biological agents that can potentially interact with the body's immune system, can actively replicate, and can even evolve during manufacture or use, but are far from unique in these regards. Many protein-based pharmaceuticals can stimulate the immune system, *e.g.*, antibiotics that lyse bacteria will release bacterial toxins in situ (Loc-Carrillo and Abedon, 2011). The use of phages as drugs may differ dramatically from pharmaceuticals/antibiotics due to differences in the phage pharmacokinetics (Payne and Jansen, 2003). Phage therapy is often complicated by additional factors and as such possess unique pharmacokinetics and pharmacodynamics that remain poorly understood (Cooper et al., 2016). The pharmacokinetics of phages are complicated due to the self-replicating nature of phages. The *in vitro* growth data for a phage cannot be directly applied to the *in vivo* situation, and the *in vivo* data for one phage cannot be transferred to another phage (Skurnik and Strauch, 2006). Critical parameters that affect phage therapy are the phage adsorption rate, burst size, latent period and initial phage dose, and also density-dependent thresholds and associated critical times should be considered (Payne and Jansen, 2001). Another parameter is the clearance rate of the phage particles from the body fluids by the mononuclear phagocyte system.

Clinical trials

Lytic bacteriophages targeting individual bacterial pathogens have therapeutic potential as an alternative or adjunct to antibiotics. Phage therapy has been used for decades, but clinical trials in this field are rare, leaving many questions unanswered as to its effectiveness for many infectious diseases (Reindel and Fiore, 2017). As a consequence, phage therapy is not used or accepted in most parts of the world (Parracho et al., 2012). The therapeutic use of phages started in Paris in 1919 when d'Herelle used oral phage preparations to treat bacterial dysentery (Kutter and Sulakvelidze, 2004). Patients treated with a single dose of phage preparation started to recover within 24 h of treatment (Abedon et al., 2011; Sulakvelidze, 2001). Not long after this, Bruynoghe published, together with his student Maisin, on the first use of phages in a phage therapeutic context (Lavigne and Robben, 2012).

In order to achieve clinical use, rigorous trials to validate safety and efficacy need to be established. The regulatory foundation for clinical studies and clinical trials in humans is to ethically establish the potential toxicity, efficacy and side effects of new drugs and to prioritize the health of the participants over the generation of results. It is equally important that sufficient data support the claim of potential benefits and that these benefits outweigh anticipated risk. Clinical studies and trials should be carried out in a scientifically correct and transparent manner, be designed to result in trustworthy data and assess the pharmacological properties of the new drug in a stepwise process adapted to available information (Cooper et al., 2016). Since bacteriophages replicate only in the presence of their host bacteria, first-in-human data with phages in healthy participants may not address safety concerns that are unique to phage-bacteria interaction in the setting of active infection, such as tolerability of therapy, immune response to therapy and endotoxin release. Assessment of phage pharmacokinetics and pharmacodynamics in otherwise healthy patients who are colonized by target strain(s) may provide some insights, such as the impact on the microbiome, but may still not predict what happens in the setting of higher bacterial burden associated with infection (Reindel and Fiore, 2017). The interpretation of pharmacokinetic data may also be affected by immune clearance of the bacteriophage (Dąbrowska et al., 2005; Merril et al., 1996).

Animal studies have generally supported the utility and safety of bacteriophage therapy against bacterial pathogens, such as *P. aeruginosa* (McVay et al., 2007; Soothill, 1994), *S. aureus* (Wills et al., 2005), vancomycin-resistant *Enterococcus faecium* (Biswas et al., 2002) and *Clostridium difficile* (Ramesh et al., 1999). Such research has culminated in preclinical and veterinary trials, such as the application of a phage cocktail to treat *P. aeruginosa* otitis in dogs (Hawkins et al., 2010).

The available phage therapy trials have primarily addressed safety issues. No adverse events have been reported so far. Intralytix has performed a clinical trial in Texas on 42 patients with chronic venous leg ulcers. These patients were treated for twelve weeks with either a saline control or a bacteriophage cocktail against *P. aeruginosa, S. aureus* and *E. coli*. Although this study was not designed as an efficacy trial, no significant differences were determined between the test and control groups for frequency of healing, but no adverse events were attributed to the study product (Rhoads et al., 2009).

The use of phages against *P. aeruginosa* has been examined in other clinical trials as well. Wright *et al*. (2009) reported the efficacy and safety of a therapeutic phage preparation (Biophage-PA). The study contained twelve patients with antibiotic-resistant *P. aeruginosa* chronic otitis who were treated with a single dose of phage preparation and followed up at 7, 21 and 42 days after treatment. The treated patients showed significant clinical improvements compared to the placebo group, although both groups still had *P. aeruginosa* at day 42 (Wright et al., 2009).

Another clinical trial was conducted in a Belgian Military Hospital on burn wound patients. Nine patients with burn wounds infected with multidrug-resistant *P. aeruginosa* or *S. aureus* were treated with a sterile-filtered cocktail of well-characterized phages, purified of endotoxin and lacking cytotoxicity (Merabishvili et al., 2009). Although only one treatment was carried out and the bacterial load remained unchanged in the patients, the authors were able to overcome important psychological hurdles to the use of phage therapy. The authors encountered numerous pitfalls, such as that the natural phage isolates were required to go through safety checks for genetically modified organisms, insurance companies put the patients in a higher risk class, and reviewers asked for conventional pharmaceutical tests of the phage cocktail, which would have cost millions of euros (Rose et al., 2014). The lack of adverse events observed in this pilot trial led to the establishment of a dedicated phage therapy center by the Belgian Army. Nevertheless, these small scale clinical trials have opened the debate discussing the regulatory frame that could fit the re-introduction of bacteriophage therapy without losing safety, quality and efficacy aspects (Debarbieux et al., 2016; Verbeken and Pirnay, 2015; Verbeken et al., 2014a, 2014b, 2016).

Although several safety studies have been conducted, not all of them take into account or report the removal of endotoxins as a crucial step in the preparation of the therapeutic phages. Endotoxin removal strategies used during these trials range from the use of specific endotoxin removal kits [*e.g.* Detoxi-endotoxin removal gel (Pierce) and Endotrap Blue (Hyglos)] (McVay et al., 2007; Merabishvili et al., 2009), CsCl ultracentrifugation (Biswas et al., 2002) or the use of custom made 0.07 μm filters (Soothill, 1994).

A large scale clinical trial using burn wound patients was setup to further evaluate the efficacy of phage therapy, Phagoburn. Phagoburn is a European Union (EU) funded project to explore the use of phage therapy to treat burn wounds infected with bacteria. Phagoburn involves institutions and hospitals in Belgium, France and Switzerland (Matsuzaki et al., 2014). Pherecydes Pharma is leading this clinical trial and developed two topical phage treatments for the trial, aimed at *E. coli* and *P. aeruginosa* infections. Initially they planned to enroll 220 patients from eleven participating hospitals. Half of these would receive phages, and the other half silver sulfadiazine, an antibacterial cream routinely used on burn infections. However, in the six months after recruitment began in July 2015, Phagoburn found just 15 eligible patients with *P. aeruginosa* infections and one with *E. coli.* Due to the difficulties in patient recruitment, the team decided to drop the *E. coli* study altogether, leaving just the intended 110-person *P. aeruginosa* study (Servick, 2016). The difficulties that the Phagoburn trial currently is having, shows the long way phage therapy trials have to accomplish in order to become a fully implemented treatment.

In order to achieve a therapeutic application and regulatory approval, a deep understanding of the phage-host interaction, phage diversity, phage dynamics and

genome function is crucial (Pires et al., 2015). Moreover, the interaction of phages with the mammalian immune system is understudied, although it might play an important role in the outcome of phage therapy.

The immunogenic properties of bacteriophages

Phage –mammalian host interactions

The human body is colonized by commensal microorganisms that encompass diverse phyla from the three domains of life: Eukarya, Archaea and Bacteria. Most of these microorganisms reside at body surfaces that are in direct contact with the environment, including the intestine, skin and upper respiratory tract. Research efforts over the past two decades have focused primarily on the bacterial component of the human microbiota and its associated genes (Duerkop and Hooper, 2013). These efforts have yielded a wealth of insight about the composition of human-associated bacterial communities, how these resident bacteria interact with the immune system and how bacteria-immune system interactions are altered in disease (Hooper et al., 2012; Lozupone et al., 2012). Recently, it has become apparent that the microbiota of healthy humans also include important numbers of viruses, termed the virobiota (White et al., 2012). Metagenomic studies have revealed that the human microbiome includes many viral genes (the virome) (Handley et al., 2012; Minot et al., 2011; Reyes et al., 2010). About 90 % of the gut virome consist of phages (Scarpellini et al., 2015).

Bacteria that inhabit the intestine and skin are generally regarded as stable residents that confer metabolic and/or immune benefits to their hosts (Turnbaugh et al., 2009). Therefore, it is reasonable to ask whether viruses can also be stably associated with healthy human tissues and whether they are able to influence the immune response. Phage populations in the intestine diversify as new members of the bacterial community are introduced, which suggests that phage diversity and bacterial diversity are linked (Breitbart et al., 2008). Although there is minimal variation of intestinal phage populations within individuals over time, there is substantial variation between individuals, even when those individuals have similar bacterial community structures (Minot et al., 2011; Reyes et al., 2010; Turnbaugh et al., 2009). It is thus interesting to consider whether phage infection of intestinal bacteria could alter community composition in ways that impact function of the immune system and influence the spread of pathogenic viruses (Duerkop and Hooper, 2013; Ivanov et al., 2008; Mazmanian et al., 2005). Part of how the microbial community influences host immunity is by limiting pathogen colonization through niche occupation and resource use. These indirect protective effects may extend to the viral members of the microbiota, of which there are an estimated 10^9 viruses per gram of faeces. Some of these viruses target mammalian cells but phages, which exclusively infect bacteria, make up the majority of this viral community (Cadwell, 2015).

Although humans are routinely exposed to phages on a daily basis, concerns persists over their immunogenicity and overall safety, presenting an additional stumbling block for the adoption of phage therapy (Cooper et al., 2016).

Non-host-derived immunity: bacteriophage adhesive to mucus model

A critical immunological barrier protecting all animals against invading bacterial pathogens but also supporting large communities of commensal microorganisms are the mucosal surfaces (*e.g.* human gut and respiratory tract)(Johansson et al., 2008; Linden et al., 2008). The mucus is predominantly composed of mucin glycoproteins, of which the amino acid backbone incorporates tandem repeats of exposed hydrophobic regions alternating with blocks bearing extensive O-linked glycosylation (Cone, 2009). By offering both structure and nutrients, mucus layers commonly support higher bacterial concentrations than the surrounding environments (Martens et al., 2008; Poulsen et al., 1994). Secretions produced by the underlying epithelium influence the composition of this microbiota (Hooper et al., 1999; Schluter and Foster, 2012; Sonnenburg, 2005). When invaded by pathogens, the epithelium may respond by increasing the production of antimicrobial agents, hypersecretion of mucin, or alteration of mucin glycosylation patterns to subvert microbial attachment (Gill et al., 2013; Jentoft, 1990; Schulz et al., 2007). Besides bacteria, phages are also present in these mucus layers. Moreover, phage concentrations are elevated in mucus relative to the surrounding environment (Barr et al., 2013).

Phages in the human gut encode a population of hypervariable proteins (Minot et al., 2012). Approximately half of these phage proteins possess the C-type lectin fold previously found in the major tropism determinant protein at the tip of the *Bordetella* phage BPP-1 tail fibers (Medhekar and Miller, 2007). These Ig-like proteins, similar to antibodies and T-cell receptors, can accommodate large sequence variation (Halaby and Mornon, 1998). Ig-like domains are also displayed in the structural proteins of many phages (Fraser et al., 2006, 2007). That most of these displayed Ig-like domains are dispensable for phage growth in the laboratory led to the hypothesis that they aid adsorption to their bacterial host under environmental conditions (Fraser et al., 2007; McMahon et al., 2005). For phage T4, it has been shown that the increased concentration of phage on mucosal surfaces is mediated by weak binding interactions between the variable Ig-like domains on the T4 phage capsid and mucin-displayed glycans (Figure 5. 2). These Ig-like domains are present in approximately one quarter of the sequenced genomes of the *Caudovirales*, and are only found in the virion structural proteins and are typically displayed on the virion surface (Fraser et al., 2006). It was thus postulated that they play an important role in the binding to bacterial surface carbohydrates during infection (Fraser et al., 2006, 2007). The predominant macromolecular constituent of mucus, the mucin glycoproteins, display hundreds of variable glycan chains to the environment that offer potential sites for binding by phage Ig-like proteins.

Furthermore, Barr *et al*. (2013) speculated that phages use the variability of the Ig-like protein scaffold to adapt to the host's ever-changing patterns of mucin glycosylation.

The presence of an Ig-like protein (highly antigenic outer capsid protein, Hoc) displayed on the capsid of T4 phage significantly slowed the diffusion of the phage on mucin solutions. Although phage particles, being inanimate and small, act as colloidal particles, they use subdiffusive motions instead of a Brownian motion. This was shown in experiments using phage T4, where the subdiffusive motions of phage T4 in mucus increases the frequency of host encounters. Thus, phage Ig-like domains that bind effectively to the mucus layer would be under a positive selection. These findings lead to the development of the bacteriophage adherence to mucus (BAM) model (Figure 5. 2), which provides a non-host-derived antibacterial defense (Barr et al., 2013; 2015).

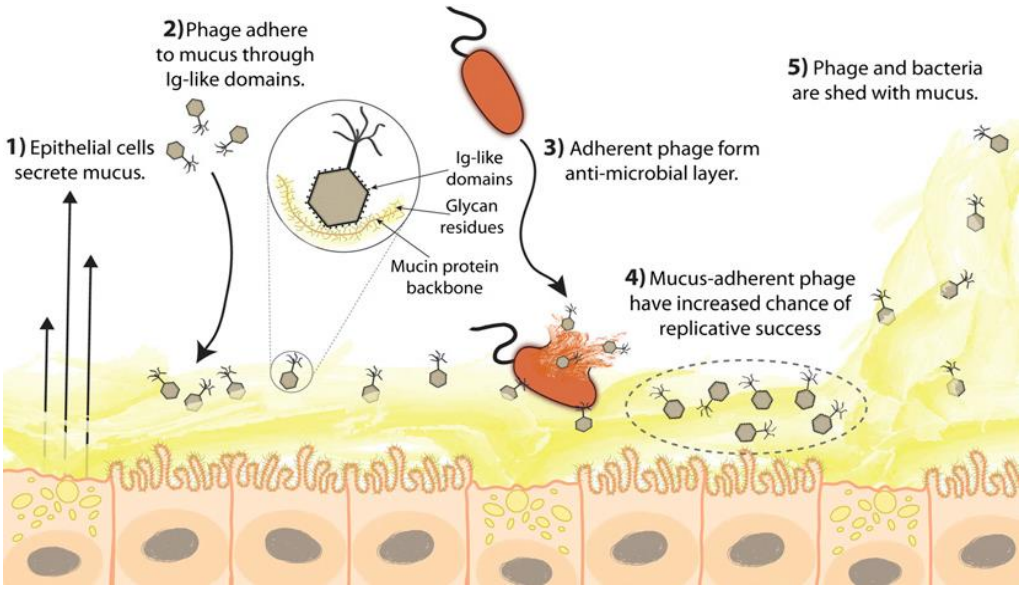


Figure 5. 2: The bacteriophage adherence to mucus (BAM model). (1) Mucus is produced and secreted by the underlying epithelium. (2) Phage bind variable glycan residues displayed on mucin glycoproteins via variable capsid proteins (e.g., Ig-like domains). (3) Phage adherence creates an antimicrobial layer that reduces bacterial attachment to and colonization of the mucus, which in turn lessens epithelial cell death. (4) Mucus-adherent phage are more likely to encounter bacterial hosts, thus are under positive selection for capsid proteins that enable them to remain in the mucus layer. (5) Continual sloughing of the outer mucus provides a dynamic mucosal environment. Figure adopted from Barr et al. (2013).

Phage transcytosis

Phages interact with non-target tissues to some extent, and at least some phages are taken up from the gastrointestinal tract into the blood. Therefore there is reason to think that such uptake can be a consequence of specific phage-to-epithelium interactions, as also appears to be the case given phage interaction with the mononuclear phagocyte system (Duerr et al., 2004; Górski et al., 2006a; Merril, 2008).

The passage of indigenous bacteria colonizing the intestine through the mucosa to local lymph nodes and internal organs is termed bacterial translocation. It is a recurrent event that happens throughout the gut and is not always a critical step in the pathology of various disorders (Guarner and Malagelada, 2003; Wiest and Garcia-Tsao, 2005). While bacterial translocation is a well-described phenomenon, little is known about the translocation of viruses. Studies have shown that the oral administration of phages to animals results in the translocation of phages to systemic tissues (Duerr et al., 2004; Hamzeh-Mivehroud et al., 2008; Keller and Engley, 1958). As such, the oral administration of phages is very effective in the treatment of alimentary tract infections in calves, lambs and piglets (Dąbrowska et al., 2005). Both the feeding and gastric lavage of animals with phages resulted in irregular but consistent recovery of phages from the blood (Keller and Engley, 1958).

Recent work from the Barr Lab demonstrated that phages can enter the body via epithelial transcytosis (Nguyen et al., 2017). This transcytosis preferentially occurs in an apical-to-basal direction and is mediated by different cell types (*e.g.* gut, lung, liver, kidney and brain cells). The transcytosis of phages across epithelial cells provides a mechanistic explanation for the occurrence of phages within the human body in the absence of disease. This work showed that the transcytosis of bacteriophages across polarized epithelial cells and into the body is a natural occurring and ubiquitous process that adds credence to the use and application of phages in a biomedical setting.

The penetration of phages in higher organisms leads to the direct contact of phages with eukaryotic cells. Therefore it is important to know whether these phages are able to interact or infect eukaryotic cells. Infection seems unlikely, because elements of the phage capsids only binds to specific molecules on the surfaces of their target bacteria. Furthermore, it is generally recognized that phages cannot infect the cells of organisms more complex than bacteria, because of major differences in key intracellular machinery that is essential for replication (Kutter and Sulakvelidze, 2004). This was illustrated by Di Giovine *et al.* (2001) who re-engineered the filamentous phage M13 to infect mammalian cells. A gene for the adenovirus penton base protein was inserted into the phage genome. This protein is originally involved in the attachment of adenoviruses to integrin receptors, internalization of viral particles, and release of the capsid from the endosome (Wickham et al., 1993; 1994). Although subsequently binding and internalization of

the re-engineered phage were observed, no multiplication of the phage was detected (Di Giovine et al., 2001).

Phage – mammalian immune response

Direct phage – mammalian interaction

In 1940, Bloch observed an accumulation of phages in cancer tissue and inhibition of tumor growth (Bloch, 1940). Later it was demonstrated that phages bind cancer cells *in vitro* and *in vivo* and attach to the plasma membrane of lymphocytes (Kantoch and Mordarski, 1958; Northrop, 1958; Wenger et al., 1978). Another study showed that phage T4 and its substrain HAP1 bind to melanoma cells and significantly inhibited the metastasis of murine B16 melanoma cells (Dąbrowska et al., 2004). The authors suggested that this interaction occurs through the binding of ß3 integrins on the target cells with the phage capsid protein gp24 (containing a KGD-amino acid motif). Eriksson *et al*. (2009) reported that genetic modification of phage M13 (designated WDC-2) led to the production of a tumor-specific phage that was able to bind 93 % of tested tumor cells (Eriksson et al., 2009). Moreover, administration of this tumor-specific phage initiated the infiltration of neutrophilic granulocytes with subsequent regression of established B16 tumors in mice (Eriksson et al., 2007, 2009). The authors observed that the mechanisms of this phage-induced tumor regression is TLR-dependent as no signs of tumor destruction or neutrophil infiltration were observed in tumors of $MyD88^{-/-}$ mice, whereby TLR signaling was abolished. Although the effects observed by these phages are not induced by a natural phage, they do indicate the possibility of phages used in different therapeutic settings or as a platform for the development of new therapeutics.

The molecular basis of these interactions is based on a Lys-Gly-Asp (KGD) tripeptide motif that forms a ligand for the ß3 integrins on cells. This mechanism was first coined by Gorski in 2003 (Gorski et al., 2003). The tripeptide motif can be found in the phage T4 structural protein gp24, which is not directly involved in the infection process of the phage. The authors suggested ways in how this hypothesis might be tested, *e.g.* using purified phages and immobilizing the phage on a plastic plate and evaluate the cell adhesion by ELISA, or confirming the bind though means of electron microscopy, or confirming the binding through the use of agents that block the ß integrin function. Unfortunately the authors have not tried any of these suggestions, leaving this hypothesis still untested. Interestingly, the adenovirus, used in the experiments of Di Giovine, binds to this integrin (Ling et al., 2002).

Cellular immune response against phages

Phage phagocytic responses

While the effects of bacteria and viruses on reactive oxygen species (ROS) activity have been described, much is not known about the effects of bacteriophages on the ROS production. A preliminary study performed by Przerwa *et al*. (2006) suggested

that phage T4 influences the phagocyte system. *In vitro* experimental models have shown that phages can diminish phagocytosis. This phenomenon may be of little significance on clinical situations, since the process of eliminating bacteria in phage-treated patients is predominantly accomplished by both phages and phagocytes (Przerwa et al., 2006). Furthermore, phage T4 inhibits the ROS production in response to pathogenic bacteria (*i.e. Escherichia coli*), and this phenomenon appears to depend on specific phage-bacterium interactions, as *P. aeruginosa* phage F-8 did not affect the ROS production induced by *E. coli* on the phagocytic cells. Although the authors conclude that the reduction of ROS production is due to the direct effect of the phage, the host-specific effect could indicate that the ROS reduction is caused by a reduction of bacteria, due to infection and lysis by the phage. This might also explain why phage T4 had an effect and not phage F-8 on the reduction of ROS induced by *E. coli.*

A more comprehensive follow-up study was conducted where polymorphonuclear leukocytes (PMN) were stimulated with one of three different R-type *E. coli* strains (*i.e. E. coli* B and *E. coli* J5, both susceptible for T4, and *E. coli* R4, resistant to T4) or with LPS derived from these three strains (Miedzybrodzki et al., 2008). The authors used these R-type strains as only the R-type LPS was able to activate ROS production by the peripheral blood PMNs, as was previously observed by Kapp *et al*. (1987). Through this setup, the authors could observe a reduction in ROS production when PMNs were stimulated with either the live bacteria or their LPS in the presence of phage T4. Moreover, this reduction was seen not only when T4 was able to infect the *E. coli* strains but also for the T4 resistant *E. coli* strain, although the T4 resistant *E. coli* strain induced a less strong ROS production compared to the T4 susceptible strains. Moreover, when only phages were added to the phagocytic cells, only a minimal ROS release was observed, indicating that phages do not directly have a pro-inflammatory response on phagocytic cells. The results provided by the authors indicate that phages can indirectly lower the pro-inflammatory responses induced by the bacteria by captering the released LPS and making it less available to PMNs (Miedzybrodzki et al., 2008).

Furthermore, when phages were administered together with the host bacteria, one recent study showed that, phages were able to stimulate bacterial phagocytosis, and this is attributed to opsonization of bacterial cells by phages. In addition, phages can remain active and infective when adsorbed onto the bacteria during intake by granulocytes (Kaur et al., 2014). Therefore, some authors have suggested that during phagocytosis, phages continue lysing the phagocytosed bacteria, helping the activity of phagocytic cells (Górski et al., 2012; Jończyk-Matysiak et al., 2015).

Phage innate immune response

The innate immune system, particularly by the components of the mononuclear phagocyte system (MPS), could be a mechanism for removing phages that are circulating in the human body (Górski et al., 2012; Navarro and Muniesa, 2017). The MPS was credited for the rapid removal of administered wild-type phage λ from the circulatory system in humans (Merril et al., 1973). Moreover Merril *et al.* (1996) were able to identify certain phage λ mutants that were capable of circumventing the MPS immune response, whereby these mutants prevailed for longer periods in the blood stream than the wild-type phage. These phage λ mutants contained a single amino acid change in the λ capsid protein E, whereby a Glu was replaced by a Lys leading to a conversion of a negative charge to a positive charge (Merril et al., 1996).

Among the mechanisms responsible for the recognition of microbial and viral structures are the TLR (Kawai and Akira, 2011). Viral nucleic acids act as PAMPs and are recognized by multiple TLRs. It could thus be postulated that phage DNA might be recognized by TLR9, which is responsible for the recognition of viral DNA (Janeway and Medzhitov, 2002), after phagocytosis of the phage.

Clear evidence concerning the cooperation of phages with the innate immune system was first provided by Tiwari *et al.* (2011), showing the necessity of a neutrophil-phage synergy in the resolution of *P. aeruginosa* infections. The authors showed that neutrophils or phages alone were not sufficient to remove a bacterial infection. The cooperation of the innate immune system and phages is necessary as the presence of neutrophils removed the phage resistant bacteria, which emerge during the phage therapeutic treatment when only a single phage is used. This was later repeated by Pincus *et al.* (2015) and Roach *et al.* (2017).

Immunological studies, *in vitro* as well as *in vivo*, on the cellular immune response induced against phages have been conducted in recent years. For example, analysis of the cytokine production of mice treated intraperitoneal for 5.5 h with four phage T4 capsid proteins (*i.e.* gp23*, gp24*, Hoc and Soc) showed that no cytokines were induced (Miernikiewicz et al., 2013). The lack of cytokine production can be explained by the early time point by which the mice were tested for the presence of cytokines or through the rapid removal of the phages from circulation. Another immunological study evaluated the cytokine production in mice induced by phage T7, after the mice were fed for 10 days with phage T7. A single dose was fed every 24 h, although an exact concentration was not provided by the authors (Park et al., 2014). Although this study had its limitations, the authors were able to demonstrate that phage T7 induced a minor increase of inflammatory cytokine production in mice, but no histological changes were observed in the tissues of the gastrointestinal organs. As no caution was taken to the presence of endotoxins, the immune responses that were observed could be, partially, due to endotoxin contamination of the used phage stock.

The effect of phages on the production of TNFα and IL6 in serum has also been studied, as well as the ability of blood cells to produce these cytokines in culture. The authors of this study used blood derived from 51 patients with long-term suppurative infections of various tissues and organs caused by drug-resistant strains of bacteria (Weber- Dąbrowska et al., 2000). These patients were treated with phages and blood samples were collected and tested for the presence of TNFα and IL6. The authors were able to observe a reduction in the production of these cytokines after long-term treatment (*i.e.* 21 days). Unfortunately, the authors were not able to show whether the observed immune response was due to the presence of the phage or due to the reduction of the bacterial count through their lysis by the phage.

Recent data indicates that *Cronobacter sakazakii* phage ES2 enhances the maturation of dendritic cells and induces the expression of IL12p40 via NF-κB signaling (An et al., 2014). This maturation presumably happens after the phagocytosis of the phage by the dendritic cells. The maturation of these dendritic cells play an important role in generating a cell-mediated immune response and subsequently in the production of phage specific antibodies.

It should be noted that many experiments performed concerning the immune response induced by phages have been carried out using phage lysates. This means that these preparations could contain remnants of bacteria lysed by the phages (*e.g.* LPS, cytosolic proteins, or membrane particles) or perhaps fragments of the host bacterial cell wall adhered to the phage tails. This makes it extremely difficult to determine the components truly responsible for the modulation of the immune response. Although, more and more studies are undertaken using highly purified phages with less than 10 EU/ml. Furthermore, only a limited amount of data is useful to assess the immunological effects induced by phages, as most studies do not differentiate between an effect induced by the phage and an effect induced by a reduction of the bacteria due to lysis by the phage.

Anti-phage antibody production

Since phages consist of tightly packed DNA or RNA and a protein coat, and since the coat consists of a relatively large number of proteins, it appears obvious that neutralizing antibodies should be produced in individuals subjected to phage therapy or exposed to naturally occurring phages. In fact, naturally occurring bacteriophages are able to induce a humoral immunity. Phage-neutralizing antibodies, that were not stimulated by phage treatment, were detected in the sera of different species (*e.g.* human) (Dąbrowska et al., 2005).

Phage immunogenicity has been employed in medicine as a test for immune competence of immunodeficient patients, *e.g.* HIV patients (Fogelman et al., 2000). In fact, immunization with bacteriophage ϕX174 has been used extensively to diagnose and monitor primary and secondary immunodeficiencies since the 1970s, without reported adverse events, even in patients in whom prolonged circulation of

the phage in the bloodstream was observed. This suggests an intrinsically low toxicity of phage ϕX174, even in patients with a compromised immune system (Ochs et al., 1971; Rubinstein et al., 2000; Shearer et al., 2001).

One of the major effects of using phages as therapeutics is a humoral response to the phage (Górski et al., 2012; Kamme, 2009; Smith et al., 1987). Soon after the discovery of phages, it was observed that antibodies against phages were produced in humans or animals (Jerne, 1952, 1956). It is indeed very easy to generate phage antisera by immunization of humans or animals with phages (Bacon et al., 1986; Górski et al., 2012; Puig et al., 2001). The humoral response does not follow a simple scheme of induction. It depends on the route of administration and on individual features of the phage. Moreover, it depends on the dose and application schedule (Dąbrowska et al., 2014a; Górski et al., 2006a, 2012; Łusiak-Szelachowska et al., 2014). The humoral response induced against phages can be devastating on the phage activity (Huff et al., 2010), but it has also been reported that the anti-phage activity of serum does not exclude a favorable result of phage therapy in humans (Łusiak-Szelachowska et al., 2014).

Initial safety studies of phage T4 performed by Bruttin and Brüssow in 2005 on humans revealed no antibody induction in phage-treated volunteers at all (Bruttin and Brüssow, 2005). Another study performed by Dąbrowska *et al.* (2014) evaluated the anti-phage antibody production against phage T4 in 50 healthy volunteers who had never been subjected to phage therapy or were involved in phage work. Of the investigated sera, 82% significantly decreased phage activity. In these positive sera, natural IgG antibodies specific to the phage proteins gp23*, gp24*, Hoc and Soc were identified. Their results clearly showed that anti-T4 phage antibodies are frequent in the human population, but it is not the highly antigenic outer capsid (Hoc) protein that induced most of the humoral response, but the antibodies specific to the major capsid protein gp23* (Dąbrowska et al., 2014a).

Recently a study concerning the production of IgG, IgA and IgM in human patients undergoing phage therapy was carried out (Zaczek et al., 2016). In this study, 20 patients were treated, for an undisclosed time, with the MS-1 phage cocktail (containing three lytic *S. aureus* phages, 676/Z, A5/80 and P4/6409) either orally and/or locally. For the majority of patients, no antibodies could be detected. For the few patients that produced elevated levels of IgG or IgM, the presence of anti-phage antibodies did not translate into an unsatisfactory clinical result of the phage therapy. The low antibody production against the phage cocktail could be due to the small time-scale by which the patients were treated. On the other hand, the elevated antibody production in a few patients could be due to a previous encounter of one of the phages used in the cocktail and the presence of an immunological memory. Unfortunately, the authors did not mention whether the antibodies were tested against individual phages or against the phage cocktail as a whole.

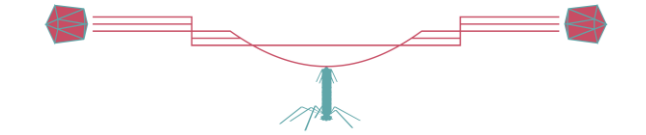
Currently it is difficult to make assumptions on the anti-phage antibody production in humans, as such studies often have contradictory results. Majewska *et al.* (2015) performed an extensive study on the antibody production against a single phage (*i.e. E.* coli phage T4) in mice over a period of 240 days. Phage T4 was orally given to mice for 100 days, followed by 112 days without phage treatment. The treatment was then repeated with the same phage up to day 240. It was demonstrated that the long term oral treatment of mice with phage T4 led to a humoral response, in contrast to previous human trials where no such responses were detected (Bruttin and Brüssow, 2005). The authors observed that this response emerged from the secretion of IgA in the gut lumen but also as an IgG production in the blood (Majewska et al., 2015). The intensity of this response and the time necessary for its induction depended on the exposure to phage antigens, which is related to the phage dose. The factor limiting phage activity in the gut was the production of specific IgA. As long as the secretory levels of IgA were low, phages remained present in the feces. When the IgA level, around day 80, increased, there were no active phages present in the feces. On the other hand, when secretory IgA decreased with time (on day 213, it became similar to its initial levels); phages could be detected again, until phage-specific IgA levels increased again.

According to the same authors, the induction of serum IgG suggested that phages could translocate from the gut lumen to the circulation. This observation is further strengthened by recent data of transcytosing phages. Furthermore, it was possible to isolate phages from murine blood after application of high phage doses (4×10^9 pfu/ml of drinking water). When lower phage doses were used, it was not possible to detect translocation of phages to the circulation, although long-lasting secondary immune response could be induced.

Besides the phage as a whole, it is interesting to evaluate the immune responses induced to individual phage proteins. Majewska *et al*. (2015) demonstrated that phage T4 Hoc protein and gp12 strongly stimulated the IgG and IgA antibody production in the blood and gut respectively, while gp23*, gp24* and Soc induced low responses.

Chapter 6

Endotoxin Purification strategies



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"The fact that we live at the bottom of a deep gravity well, on the surface of a gas covered planet going around a nuclear fireball 90 million miles away and think this to be normal is obviously some indication of how skewed our perspective tends to be."

Douglas Adams, The Salmon of Doubt: Hitchhiking the Galaxy One Last Time

Abstract

Bacterial endotoxins have high immunogenicity. Phage biology studies as well as therapeutic phage applications necessitate highly purified phage particles. In this study, we compared combinations of seven different endotoxin removal strategies and validated their endotoxin removal efficacy for five different phages (*i.e.* four *Pseudomonas aeruginosa* phages and one *Staphylococcus aureus* phage). These purification strategies included Endotrap HD column purification and/or CsCl density centrifugation in combination with Endotrap purification, followed by organic solvent (1-octanol), detergent (Triton X-100), enzymatic inactivation of the endotoxin using alkaline phosphatase and CIM monolithic anion exchange chromatography. We show that CsCl density purification of the *P. aeruginosa* phages, at an initial concentration of 10^{12} - 10^{13} pfu/ml, led to the strongest reduction of endotoxins, with an endotoxin removal efficacy of up to 99 %, whereas additional purification methods did not result in a complete removal of endotoxins from the phage preparations and only yielded an additional endotoxin removal efficacy of 23 to 99 %, sometimes accompanied with strong losses in phage titer.

Introduction

The purification of bacteriophage particles is important for two reasons: either to investigate the phage particle on its own (*i.e.* phage biology studies) or for therapeutic application of phages, which is currently undergoing a resurgence (Adhya et al., 2014; Dąbrowska et al., 2014b; Ly-Chatain, 2014; Międzybrodzki et al., 2012; Thiel, 2004; Vandenheuvel et al., 2015). When phages are propagated on Gram-negative bacterial hosts, endotoxins or lipopolysaccharides (LPS) have to be removed from these preparations. Endotoxins are part of the Gram-negative bacterial outer membranes and play an important role in the organization and stability of the bacterial cell (Ki et al., 1994). Bacterial endotoxins are well known for their immunogenic, pro-inflammatory and pyrogenic effects (Aderem and Ulevitch, 2000). In conditions where the body is exposed to endotoxins excessively or systemically, a systemic inflammatory reaction can occur, leading to multiple pathophysiological effects such as endotoxin shock, tissue injury and death (Anspach, 2001; Erridge et al., 2002; Ogikubo et al., 2004). Therefore, when phages are prepared for therapeutic purposes, it is crucial that different bacterial contaminants are removed which affect the efficacy and safety of the administration during phage therapy. The maximal level of endotoxins for intravenous applications of pharmaceutical and biological products is set at 5 endotoxin units (EU), *i.e.* 500 pg of endotoxins, per kg of body weight per hour (Daneshian et al., 2006). Additionally, bacterial endotoxins may also interfere with phage biology studies, especially when trying to establish the interaction of phages with the immune system.

Several strategies have been described for the removal of endotoxins from phage preparations. Here we compared different endotoxin removal strategies for the removal of endotoxins from five phages, *i.e.* four Gram-negative *Pseudomonas aeruginosa* phages and one Gram-positive *Staphylococcus aureus* phage (Table 6.

1). The *S. aureus* phage forms a negative control for the endotoxin determination assay, as this phage is grown on a Gram-positive host that produces no endotoxins. Strategies were compared, taking into account the efficacy in removing endotoxins in relation to their effect on the phage titer yield.

Table 6. 1: Different phages used in the different purification strategies. For each phage, taxonomical family and bacterial host strain are presented, as well as the titer of the phage lysates, before application of any endotoxin removal techniques.

Bacteriophage name	Phage family	Bacterial Host	Titer (pfu/ml)	Reference	Isolated by	Isolation date
P. aeruginosa phage PNM	*Podoviridae*	*P. aeruginosa* strain 573	1.8×10^{13}	Merabishvili et al. (2009)	N. Lashki & M. Tediashvili	1999
P. aeruginosa phage LUZ19	*Podoviridae*	*P. aeruginosa* strain 573	5.0×10^{13}	Lammens et al. (2009)	P.J. Ceyssens	2006
P. aeruginosa phage GE-vB_Pae-Kakheti25	*Siphoviridae*	*P. aeruginosa* strain 573	2.5×10^{12}	Karumidze et al. (2012)	N. Kvatadze	2012
P. aeruginosa phage 14-1	*Myoviridae*	*P. aeruginosa* strain 573	3.6×10^{12}	Ceyssens et al. (2009)	V. Krylov	2000
S. aureus phage ISP	*Myoviridae*	*S. aureus* strain ATCC 6538	8.0×10^{13}	Vandersteegen et al. (2011)	Unknown	1920 - 1930

Results and discussion

In this study, we evaluated the endotoxin removal efficacy of seven purification strategies (Figure 6. 1). To determine which strategy has the best endotoxin removal capacity, in combination with the minimal amount of phage loss, we calculated the 'endotoxin removal efficacy', defined as the ratio of the endotoxin units (EU) per plaque forming unit (pfu) multiplied by the phage recovery of the purified sample and the original sample subtracted from one (Table 6. **2**). The endotoxin quantification by Endozyme was validated by endotoxin quantification by means of Endosafe-PTS, for a selected number of samples. Both detection methods gave similar results within the same order of magnitude.

The endotoxin removal strategies include either (1) Endotrap HD column purification alone (Merabishvili et al., 2009) (φET), or (2) CsCl density gradient ultracentrifugation alone (Lavigne et al., 2009) (φC) or (3) followed with Endotrap HD purification (φCET), and φET or φCET followed by either (4) organic solvent (1-octanol; Szermer-Olearnik and Boratyński, 2015) treatment (OS), (5) detergent (Triton X-100; Marcus and Prusky, 1987; Petsch and Anspach, 2000) treatment (TX), (6) enzymatic inactivation of the endotoxin using alkaline phosphatase (Bentala et al., 2002) (AP) or (7) anion-exchange chromatography (CIM DEAE disk column (CIM); Adriaenssens et al., 2012). We opted for these combined strategies, to compare the efficacy of purifying raw phage lysates (φET) versus CsCl-purified phages (φCET). As expected, phage ISP preparations from a Gram-positive host did not show any detectable endotoxin levels before or after any of the purification strategies. The four *P. aeruginosa* phages contained between 31,020 and 7,465,000 EU/ml. This concentration was measured after purification of phage lysates with Endotrap HD and therefore may initially be higher.

Figure 6. 1: Schematic representation of the different endotoxin strategies used, starting from different phage preparations. Phage lysates were obtained by the overlay-agar method. Part of this phage lysate was used either for (A) endotoxin removal using Endotrap HD (φET) or (B) further purified through CsCl density centrifugation followed by Endotrap HD (φCET). These preparations were further treated for the removal of endotoxins through different strategies: (OS) Organic solvent: 1-octanol; (TX) detergent treatment: Triton X-100; (AP) Enzymatic inactivation of endotoxins: alkaline phosphatase; or (CIM) anion-exchange chromatography: CIM DEAE disk column (only performed on two phages).

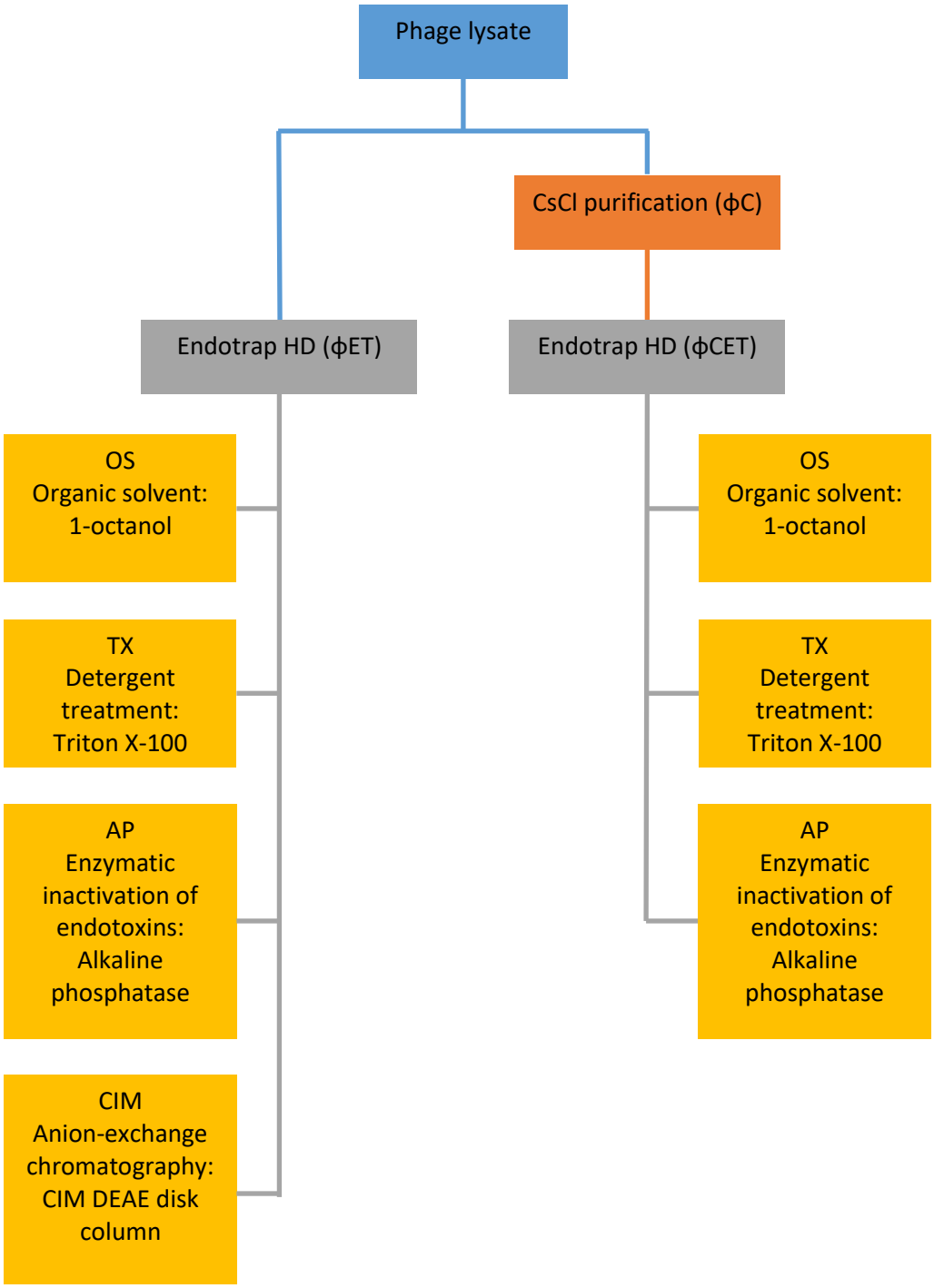
Phage lysate
CsCl purification (ϕC)
Endotrap HD (ϕET)
Endotrap HD (ϕCET)
OS
Organic solvent:
1-octanol
TX
Detergent
treatment:
Triton X-100
AP
Enzymatic
inactivation of
endotoxins:
Alkaline
phosphatase
CIM
Anion-exchange
chromatography:
CIM DEAE disk
column
OS
Organic solvent:
1-octanol
TX
Detergent
treatment:
Triton X-100
AP
Enzymatic
inactivation of
endotoxins:
Alkaline
phosphatase

Table 6. 2: Endotoxin removal efficacy. This is defined as the ratio of the endotoxin units (EU) per plaque forming unit (pfu) multiplied by the phage recovery of the purified sample and the original sample subtracted from one.

Phage name	Lysate (φET)	CsCl	CsCl	(φET)	(φCET)	(φET)	(φCET)	(φET)	(φCET)	(φET)
		(φC)	(φCET)	OS		TX		AP		CIM DEAE
***P. aeruginosa* phage PNM**	N.A.	99.42%	63.09%	99.98%	23.40%	93.21%	56.38%	-9.62%	17.17%	98.15%
***P. aeruginosa* phage LUZ19**	N.A.	18.42%	83.29%	99.68%	-16.55%	N.D.	83.92%	2.84%	20.09%	40.39%
***P. aeruginosa* phage GE-vB_Pae-Kakheti25**	N.A.	99.68%	94.56%	92.97%	63.36%	93.71%	96.95%	-5.83%	22.14%	N.D.
***P. aeruginosa* phage 14-1**	N.A.	69.33%	64.97%	63.83%	63.29%	92.86%	99.69%	-38.96%	7.25%	N.D.
***S. aureus* phage ISP**	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.D.

Negative values indicate an enrichment of endotoxins. N.D.: not determined. N.A.: not applicable. Dark gray indicates endotoxin removal efficiencies larger than 99%, whereas light gray indicates endotoxin removal efficiencies between 80 – 99 % and white indicates below 80. CsCl: CsCl density centrifugation, OS: organic solvent, TX: Triton X-100, AP: alkaline phosphatase, CIM DEAE: Convective Interactive Media.

However, it is possible that these values correspond largely to the initial order of magnitude of the endotoxins present in the crude phage preparations, because the removal efficacy of the Endotrap HD purification, starting from the CsCl purified phages, was only between 63.09 – 94.56 % (Table 6. **2**). Cooper *et al*. (2014) showed that crude *P. aeruginosa* phage preparations contained more than 10^5 EU/ml, which was in correspondence with our quantification of preparations that were purified only once with Endotrap HD. Dufour *et al*. (2016) recently described that three to five consecutive rounds of Endotrap HD-based endotoxin removal, starting from CsCl purified phages, led to a further reduction of the endotoxin concentration to below 0.5 EU/ml, but this is a time-consuming strategy with a variable and phage-dependent outcome.

Organic Solvent (OS)

The organic solvent extraction was described by Szermer-Olearnik and Boratyński (2015) and is based on the principle that endotoxins partition favorably in the organic phase, while the molecules of interest (in our case phages) are retained in the aqueous phase. Our results show that the organic solvent strategy has an endotoxin removal efficacy between 63.83 and 99.98 % for φET and between 23.40 and 63.36 % for the φCET samples. The only exception is *P. aeruginosa* phage LUZ19 where there was an enrichment of endotoxins after the 1-octanol treatment, *i.e.* an endotoxin removal efficacy of -16.55 %. This enrichment could be explained by a potential release of endotoxins bound to the phage particle, which when bound are not detectable in the endotoxin detection assay.

The organic solvent strategy led to the strongest reduction of endotoxins in the *P. aeruginosa* phage PNM φET and phage LUZ19 φET preparations, with an endotoxin removal efficacy of 99.98 and 99.68 % respectively. This was only accompanied with a reduction of the *P. aeruginosa* phage PNM with two orders of magnitude. For both phages, a strong reduction in phage titer of up to five orders of magnitude difference was observed.

Detergent: Triton X-100

The endotoxin removal strategy using detergents such as Triton X-100 has been well-established. According to Petsch and Anspach (2000), endotoxins can be removed using a two-phase extraction, employing detergents such as those of the Triton series. An endotoxin removal efficacy of up to 93.21 % and 99.96 % could be observed for the φET and φCET phage samples, respectively (Table 6. 2). The use of activated charcoal for the removal of Triton X-100 led to a reduction in the endotoxin concentration after three consecutive rounds. Around 6.29 – 7.14 % endotoxins remained for the φET preparations and between 0.31 – 43.6 % endotoxins remained for the φCET preparations. However, the activated carbon was not able to remove all of the Triton X-100. This became apparent when the samples became viscous when stored at 4 °C for prolonged time. The phage recovery for the φET was between 0.07 – 17.20 % and for the φCET it was between 1.80 – 52.22 %

(Table 6. 3), indicating that the Triton X-100 strategy has an effect on the phage activity.

Enzymatic inactivation of endotoxins: alkaline phosphatase

An alternative method for the removal of endotoxins is enzymatic inactivation of endotoxins by the removal of the phosphate group of the lipid A fraction by means of alkaline phosphatase (Bentala et al., 2002). Consequently, the treated endotoxin becomes immunologically inactive and should not be detected in a classical Limulus Amebocyte Lysate (LAL) assay or by recombinant factor C (rFC) assays as used in this study. However, the treatment of the φET and φCET phage preparations with alkaline phosphatase had very low endotoxin removal efficacies (*i.e.* lower than 20 %; Table 6. 2) and did not lead to a reduction in the endotoxin concentration. In addition, this method had a negative impact on the number of infectious phage, dropping 2 – 4 orders of magnitude (Table 6. 3).

Although this strategy has been described for the inactivation of purified endotoxins, when it was applied on phage samples, there was no reduction in the endotoxin concentration. This might indicate that either the phages inhibit the enzymatic activity of the alkaline phosphatase or that the phages, through the binding of the endotoxins, hide the phosphate groups on the lipid A part. Consequently, this can result in the endotoxins not being completely inactivated by the enzyme while still being detectable in the endotoxin detection assay.

Anion-exchange purification: CIM DEAE disks

The final strategy that was evaluated was the anion-exchange purification using CIM DEAE columns. This technique was previously described for its application in the purification and concentration of phages (Adriaenssens et al., 2012), but the authors did not evaluate the endotoxin removal potential. In this strategy, the phages are retained on the column through ionic interactions. This purification protocol was only performed for the φET preparations of *P. aeruginosa* phages PNM and LUZ19. Although the two phages that were purified by this method (*i.e. P. aeruginosa* phage PNM and LUZ19) have a high sequence similarity, the endotoxin removal efficacy varied between these two phages (*i.e.* 98.15 and 40.39 %, respectively; Table 6. 2). A strong reduction in phage titers from 10^{13} to 10^{11} pfu/ml for both the *P. aeruginosa* phages PNM and LUZ19 was observed (Table 6. 3). The limited reduction in endotoxin concentration after the anion-exchange purification could be explained by the fact that endotoxins also have the ability to interact with anion-exchange columns. According to Hou and Zaniewski (1990), the capacity of endotoxin removal by anion-exchange matrices through charge interaction depends on the number of available positively charged groups existing in the matrices. They observed a maximum adsorption of endotoxins at pH 6.8 when DEAE columns were used, and at pH 8.0 when QA columns were used. They also found that the endotoxin adsorption was found to be unaffected at up to 0.2 M salt concentration (Hou and Zaniewski, 1990a). We found that both phages were retained at the

column at a pH of 7.5 and eluted at a NaCl concentration above 0.6 M. Therefore, the removal of endotoxins without loosening the phages from the column is cumbersome. Hence, the endotoxins co-elute with the phages minimizing the ability to obtain endotoxin free phage preparations. The reduction in phage titers results from the phage binding capacity of the columns, which is phage-dependent.

Conclusions

Starting from between 2.5 x 10^{12} and 8 x 10^{13} pfu/ml of four different *P. aeruginosa* phages (Table 6. 3), contaminated by 31,020 and 7,465,000 EU/ml, we found that CsCl density gradient ultracentrifugation established an endotoxin removal efficacy between 18.42 and 99.68 % (Table 6. 2) while reducing the number of phages with maximum of two orders of magnitude (Table 6. 3). Further endotoxin removal of these CsCl preparations with Endotrap, OS, TX, AP or CIM did reduce endotoxins further with a maximum endotoxin removal efficacy of 99.9 %, whereas several of these additional treatments were detrimental for the phage titer, which was even reduced to 0.0004 % for the *P. aeruginosa* phage PNM φCET OS treated sample.

From our comparative study, it becomes clear that it is hard to achieve complete removal (*i.e.* ≥ 99.99 %) of endotoxins from a phage sample. For therapeutic purposes, only 5 EU/ml/kg/h can be present in the samples for intravenous applications (Daneshian et al., 2006). We clearly find that the removal of large volumes of endotoxins is easier than the removal of small residual endotoxins, as the endotoxin removal efficacy of the different procedures starting from the phage lysate (φET) preparation is much higher than those form the CsCl purified (φCET) preparations (Table 6. 2). Unfortunately, we were not able to obtain a universal strategy that could be used for the removal of endotoxins from any given phage preparation. Therefore, each phage needs to be evaluated individually for the optimal strategy for the removal of endotoxins, taking into account the potential drop in phage titers. The CsCl purification (φC) seems to have the highest efficacy in removing endotoxins. Although this technique might not be suitable for all applications such as phage therapy where high throughput and up scaling is a must, this strategy might be important for phage biology studies in which endotoxin contamination may result in confounding effects.

Table 6. 3: Titers of single phage after each specific endotoxin removal strategy.

Phage name	Lysate (ϕET)	CsCl (ΦC)	CsCl (ϕCET)	ϕET	ϕCET OS	ϕET	ϕCET TX	ϕET	ϕCET AP	ϕET CIM
	pfu/ml			%						
***P. aeruginosa* phage PNM**	1.80E+13	3.30E+13	3.00E+13	2.06	0.0004	2.00	2.10	2.61	1.57	2.44
***P. aeruginosa* phage LUZ19**	5.00E+13	5.00E+11	5.00E+11	34.00	0.0024	N.D.	14.00	0.02	6.00	0.62
***P. aeruginosa* phage GE-vB_Pae-Kakheti25**	2.50E+12	9.00E+10	9.00E+10	3.20	130.00	17.20	52.2	16.4	8.89	N.D.
***P. aeruginosa* phage 14-1**	3.60E+12	5.00E+12	5.00E+12	26.11	31.00	1.11	1.80	9.44	30.6	N.D.
***S.aureus* phage ISP**	8.00E+13	3.00E+11	3.00E+11	0.03	10.00	0.07	22.8	0.005	0.53	N.D.

All phage titers are expressed as pfu/ml or phage recovery percentage, the amount of phages remaining after the purification as compared to the original sample (phage lysate after Endotrap HD (φET) or CsCl purified phage followed by Endotrap HD purification (φCET), respectively). N.D.: not determined. Dark gray indicates phage recovery above 90 %, light gray between 20 – 90 % and white below 75 %. CsCl: CsCl density centrifugation, OS: organic solvent, TX: Triton X-100, AP: alkaline phosphatase, CIM DEAE: Convective Interactive Media.

Since the phage titers used for phage therapy are usually around 10^8 pfu/ml (Merabishvili et al., 2009), the phage preparations can be further diluted leading to a further drop of the endotoxin concentration (ranging from 0.0002 to 316 EU/ml for 10^8 pfu/ml phage preparations; Figure 6. 2) and subsequent safe use. Therefore, labor-intensive endotoxin removal strategies should not be necessary for therapeutic phage preparations, knowing that the dilution of high titer phage preparations would be sufficient. Unfortunately, dilution of the phage preparations is not always possible when performing phage biology studies such as evaluating the immunological properties of phages on the mammalian immune system, where high phage titers might be advised (Biswas et al., 2002; Miernikiewicz et al., 2013). Although this study has as a limitation that no technical replicates were obtained for the different endotoxin removal strategies, our results do indicate that the endotoxin removal is phage dependent, and thus needs to be evaluated individually for each phage. To obtain a complete removal of endotoxins, a combination of strategies could be used, such as treating the phage lysate with Triton X-100 followed by a purification and concentration of the phages through means of anion-exchange (*i.e.* DEAE disks) or by using consecutive rounds of Endotrap HD endotoxin removal after CsCl density centrifugation, as suggested by Dufour et al. (2016).

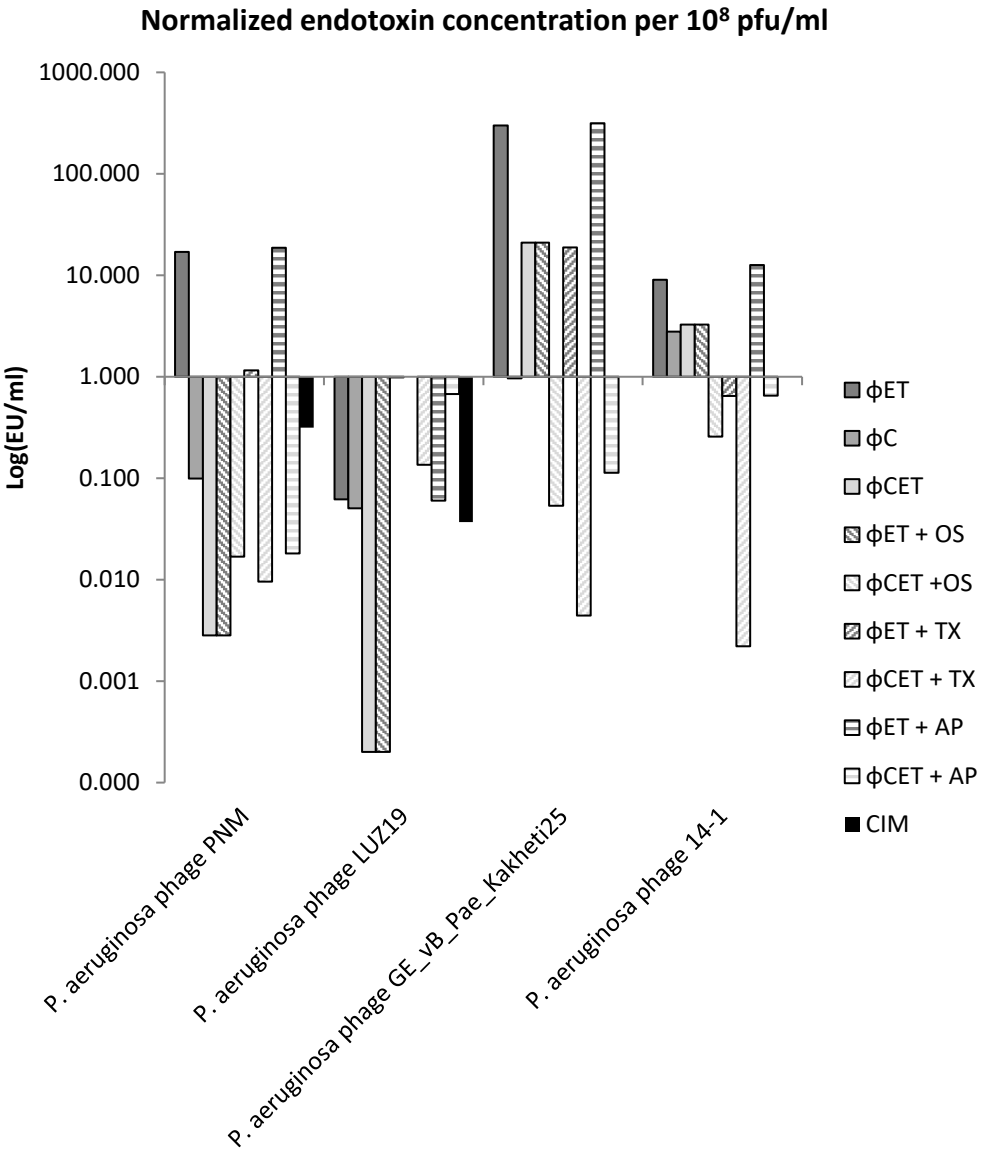

Figure 6. 2: Final endotoxin concentration (EU/ml) present after different purification strategies for four different *Pseudomonas aeruginosa* phages, normalized against a phage therapeutic titer (*i.e.* 10^8 pfu/ml). φET: lysates after Endotrap HD, φC: CsCl purification, φCET: φC after Endotrap, OS: Organic Solvent, TX: Triton X-100, AP: Alkaline Phosphatase, CIM DEAE: Anion Exchange using CIM DEAE disks.

Material and methods

Bacteriophage and bacteria

Phage propagation

Bacteriophage stocks (Table 6. 1) were prepared using the double-agar overlay method as described in Merabishvili *et al.* (2009). Briefly, one ml of the phage preparation containing 10^6 plaque forming units (pfu) of bacteriophages was mixed with 3 ml of molten (45 °C) Lysogeny Broth (LB) (Becton Dickinson, Erembodegem, Belgium) top Bacto agar (0.6 %) (Becton Dickinson) and 100 µl of the host strain suspension (end concentration of 10^7 cfu/ml) in a sterile 14 ml tube (Falcon, Becton Dickinson). This mixture was plated onto freshly made 90 mm diameter Petri dishes (Plastiques Gosselin, Menen, Belgium), filled with a bottom layer (20 ml) of 1.5 % LB agar, and incubated aerobically at 32 °C for 16 h. Subsequently, 200 µl of chloroform was added to the lids of the Petri dishes and the inverted plates were further incubated at 4 °C for 1 h. The top layer of the double-agar layer was scraped off using a sterile Drigalski spatulum and transferred to a sterile 14 ml tube.

The harvested phages were centrifuged for 20 min at 6,000 x *g* at 4 °C. The supernatant was aspirated using a sterile 10 ml syringe (BD Plastipak, Becton Dickinson) with a 30 G sterile needle (BD Microlance 3, Becton Dickinson) and passed through a 0.22 µm membrane filter (Sartorius Stedim, Zellik, Belgium). The filtrate was subsequently centrifuged at 35,000 x *g* for one hour. The phage pellet was resuspended in 5 ml saline and stored at 4 °C overnight before determining the phage titer. Preferably, the titer of the phage lysate should be checked at least one day later according to the above described procedures. This will allow phage particles that may have clumped together during centrifugation steps to disengage (Kutter and Sulakvelidze, 2004).

Phage titer determination

The bacteriophage titer was determined by assaying decinormal serial dilutions (log(0) to log(-12)) of the bacteriophage suspension with the overlay method (Merabishvili et al., 2009). One ml of each dilution was mixed with 3 ml of molten (45 °C) LB 0.6 % top Bacto agar and the host strain (end concentration of 10^7 cfu/ml) in a sterile 14 ml tube. This mixture was plated in triplicate onto 90 mm diameter Petri dishes, filled with a bottom layer of 1.5 % LB agar, and incubated for 16 h at 37 °C. To determine the original bacteriophage concentration, plates with one to 100 distinguishable homogenous plaques were counted. The mean was then calculated for the triplicate plates.

Endotoxin removal strategies

All manipulations were carried out using endotoxin-free reagents. Figure 6. 1 gives a schematic representation of the different purification strategies used.

CsCl purification

Phage lysates particles were further purified and concentrated by means of ultracentrifugation (104,000 x *g*, 4 °C) for 3.5 h in a CsCl (PanReac AppliChem, Darmstadt, Germany) gradient with densities of 1.33 to 1.70 g/cm^3 in a swigging bucket centrifuge as described by Lavigne et al. (2009). The resulting high-titer phage suspension (ca. 3 – 4 ml) was dialyzed with a Slide-A-lyzer Mini Dialysis device (10,000 MWCO, Thermo Scientific, Hudson, NH) three times for 30 min at 4 °C against 1 l of saline to remove residual CsCl. The CsCl-purified phage samples (φC) were stored at 4 °C and the phage titer was determined on the following day.

Two-phase extraction

Organic solvent (OS) treatment: 1-octanol. Phage φET and φCET preparations were treated with an organic solvent (1-octanol; Sigma-Aldrich, Munich, Germany), as described by Szermer-Olearnik and Boratyński (2015). Briefly, 500 µl of the phage preparation (either phage lysate (φET) or CsCl purified phages (φCET)) was transferred to a 1.7 ml Eppendorf tube. Subsequently, $MgCl_2$ (Sigma-Aldrich) was added to a final concentration of 0.02 M. This mixture was incubated for 1 h at 4 °C. After incubation, 1-octanol (40 % v/v) was added and mixed overnight by inverting in a vertical rotator at room temperature. The mixture was incubated for 1 h at 4 °C prior to centrifugation at 4,000 x *g* for 10 min. The upper 1-octanol phase was removed and the lower aqueous phase was transferred to Slide-A-lyzer Mini Dialysis device (10,000 MWCO). Dialysis was performed against ethanol (25 %) for five subsequent rounds (one overnight incubation and four 2 h incubations). Subsequently, the samples were dialyzed against endotoxin-free saline for four rounds (one overnight and three incubations of two hours). The purified phage solution was stored overnight at 4 °C and the phage titer was determined on the following day.

Detergent treatment: Triton X-100. The Triton X-100 removal of endotoxins is based on general protocols for the removal of endotoxins from protein preparations, as described by Petsch and Anspach (2000). Above the critical micelle concentration of some detergents, endotoxins are trapped in a micellar structure by non-polar interactions of the alkyl chains of lipid A and the detergent and are consequently separated from the water phase. Detergents of the Triton series show a miscibility gap in aqueous solution. Above a critical temperature, the so-called cloud point, micelles aggregate to droplets with very low water content, thus forming a new phase. Endotoxins remain in the detergent-rich phase. The cloud point of Triton X-114 is at 22 °C, which is advantageous when purifying proteins. It requires mixing of the endotoxin-containing protein solution in the cold (usually at 4 °C) and allows separation of the two phages at temperatures of 22 °C or above. In contrast, the cloud point for Triton X-100 is at 75 °C, which is not acceptable for most proteins or phages, because high temperatures might lead to inactivation. In the classical protocols, Triton is removed by centrifugation. This has as downside that it is impossible to remove all the Triton present in the sample. Alternatively, Triton can

be removed by using activated charcoal (Marcus and Prusky, 1987), which has as an additional advantage that it is not necessary to use a two-phase system, which makes it possible to use Triton X-100 (which has, due to its higher cloud point, the advantage of being used at room temperature). A total volume of 200 µl of the phage solution (*i.e.* φET or φCET) was transferred to a 1.7 ml Eppendorf tube. To this solution, 3 % (v/v) Triton X-100 (Sigma-Aldrich) was added and incubated for 30 min at room temperature while shaking at 750 rpm. After incubation, 12 % activated carbon (Sigma-Aldrich) was added to remove the Triton X-100 (Marcus and Prusky, 1987). An additional 30 min of incubation was performed at room temperature while shaking at 750 rpm. The solution was centrifuged at maximum speed for 1 min, after which it was passed through a 0.45 µm membrane to remove residual activated carbon. All steps were repeated for an additional two rounds, *i.e.* a total of three Triton X-100 treatments. The purified phage solution was stored overnight at 4 °C and the phage titer was determined on the following day.

Enzymatic inactivation of endotoxins: alkaline phosphatase (AP)

The inactivation of endotoxins through enzymatic degradation by means of alkaline phosphatase was described by Bentala et al. (2002). Briefly, alkaline phosphatase (rSAP (1,000 U/ml;7.5 U/reaction of 300 µl), New England Biolabs, New England, MA) together with the CutSmart buffer (New England Biolabs; as described by the manufacturer) was added to the phage sample and incubated for 60 min at 37 °C. Subsequently the enzyme was inactivated by heating the solution for 5 min at 65 °C. Following endotoxin inactivation, the titer of the phage solution was determined.

Adsorption techniques

Endotrap HD. Endotrap HD is an affinity chromatography based strategy using bacteriophage-phage derived proteins that are fixed on the column matrix and bind endotoxins with a high affinity and specificity. According to the manufacturer, Endotrap HD is able to remove endotoxins from protein, peptides, antibodies, RNA/DNA, antigens and plant extract samples with an endotoxin removal efficiency of 99.99 %. It also is claimed to have a wide pH range (4 – 10) of activity and that high salt concentrations do not affect the endotoxin removal capacity. The phage lysates (φET) or CsCl purified phage lysates (φCET) were further purified from endotoxins using the commercially available kit Endotrap HD (Hyglos, Bernried am Starnberger Seen, Germany), according to the instructions of the manufacturer. Briefly, 3 ml of the phage preparations were transferred to a sterile 15 ml Falcon tube (Becton Dickinson) and $CaCl_2$ (Sigma-Aldrich) was added to a final concentration of 0.001 M. Prior to the addition of the phages to the columns, the columns were activated by the addition of 3 ml regeneration buffer. The columns were drained out completely before repeating the addition of the regeneration buffer. Subsequently, 3 ml of the equilibration buffer was added and the columns were drained out completely before a second wash with the equilibration buffer was performed. Finally, the phage samples were added to the column and the eluate was collected in a sterile 15 ml Falcon tube (Becton Dickinson). The columns were then

regenerated by washing them twice with 3 ml regeneration buffer. The columns could then be used for a second purification round or be stored by adding 1 ml of the storage buffer supplemented with 0.02 % sodium monoazide (Sigma-Aldrich). Following endotoxin removal, the titer of the phage solution was determined.

Anion-exchange chromatography: Convective Interactive Media (CIM) disk. Phage purification using monolithic anion-exchange chromatography with CIM DEAE disk columns (BIA Separations, Ljubljana, Slovenia) was carried out, basically as described by Adriaenssens et al. (2012). Briefly, prior to phage purification, the complete chromatography set up (Äkta FPLC system (GE Healthcare, Little Chalfont, UK with a P900 pump system) was flushed with 1 M NaOH. The loading column was washed three times with endotoxin-free water. Next, approximately 10 ml of one of the high titer phage preparations was loaded onto the chromatography set up. Pump A contained Tris-HCl (20 mM, pH 7.5) buffer whereas pump B contained Tris-HCl (20 mM, pH 7.5) with NaCl (2 M) buffer. Prior to the elution of the phages from the CIM DEAE disk column, the column (containing the phages) was washed with the Tris-HCl (20 mM, pH 7.5) buffer. Elution of the phages from the CIM DEAE disk columns was achieved by washing with an increasing percentage of the Tris-HCl (20 mM, pH 7.5) with NaCl (2 M) buffer relative to the Tris-HCl (20 mM, pH 7.5) buffer.

Endotoxin quantification

Quantification of the endotoxin concentrations in the differently treated phage preparations was performed using two different commercially available methods. Prior to the endotoxin determination, all phage samples were diluted using Endotoxin-Free Dulbecco's PBS (Millipore, Darmstadt, Germany).

The endotoxin removal efficacy of each purification approach was calculated by first determining the amount of endotoxins per plaque forming unit (pfu) by dividing the endotoxin concentration by the phage titer for each preparation (1).

$$\text{Normalized endotoxin content} = \frac{EU/ml}{pfu/ml} \quad (1)$$

Subsequently the normalized endotoxin content of each sample was determined by multiplying the endotoxins per pfu with the phage recovery (2), *i.e.* the phage titer of the purified preparation divided by original preparation.

$$\text{Phage recovery} = \frac{(pfu/ml)_{purified\ sample}}{(pfu/ml)_{original\ sample}} \quad (2)$$

The endotoxin removal efficacy (3) was then calculated by dividing the normalized endotoxin concentration of the purified sample by the normalized endotoxin concentration of the original sample (*i.e.* φET or φCET) and subtracting this value from 1.

$$\text{Endotoxin removal efficacy} = 1 - \frac{\left[\left({}^{EU/ml}/_{pfu/ml}\right)\times\text{phage recovery}\right]_{purified\ sample}}{\left[\left({}^{EU/ml}/_{pfu/ml}\right)\times\text{phage recovery}\right]_{original\ sample}} \quad (3)$$

EndoZyme recombinant Factor C (rFC) Assay

According to the manufacturer, EndoZyme (Hyglos, Bernried am Starnberger See, Germany) is an endpoint fluorescent microplate assay intended for *in vitro* quantitative determination of endotoxin (lipopolysaccharides, LPS) in pharmaceuticals and biological substances as well as for medical device testing using recombinant factor C (rFC). The enzymatically active rFC is activated by trace amounts of endotoxins. It is capable of binding both free and bound LPS/lipid A (the biologically potent moiety of LPS) with high affinity. Being at the initial step of the coagulation cascade, Factor C functions as a very sensitive and specific biosensor of endotoxins, capable of detecting pictogram to nanogram levels of endotoxins (Ding and Ho, 2010). Endozyme is able to reliably detect endotoxins within the range of 0.005 to 50 EU/ml. The phage samples were diluted until their endotoxin concentration fell within this range. The assay was performed as described by the manufacturer.

Endosafe-PTS

According to the manufacturer, the Endosafe-PTS (Charles River, CA) is a rapid, point-of-use test system that provides quantitative LAL results within 15 minutes. It is a miniaturized version of the LAL assay using rFC. It is able to detect endotoxins within the range of 0.005 to 10 EU/ml. The endotoxin determination was performed by the VIB Protein Service Facility (Ghent University) as described by the manufacturer.

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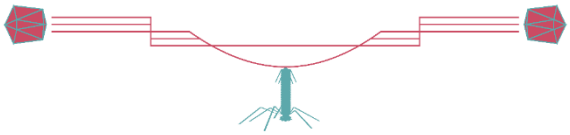
Chapter 7

Transcriptome Analysis



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"Everything must be made as simple as possible.
But not simpler."

Albert Einstein

Abstract

Bacteriophages, the most abundant biological entities on Earth, are well known for their therapeutic applicability, but their effect on the human immune system is less known. Here we describe an RNA transcriptome analysis of peripheral blood mononuclear cells stimulated either with *P. aeruginosa* phage PNM or its bacterial host *P. aeruginosa* strain 573. *P. aeruginosa* phage PNM was able to increase the production of IL10, IL6, SOCS1, SOCS3, CXCL2, CXCL3, CXCL6 and decrease the production of lysozyme, HLA-DMA, HLA-DMB, HLA-DRB1 and HLA-DRB4, CCL17, CCR1, CCR2 and CCR5. *P. aeruginosa* strain 573 on the other hand was able to down-regulate the production of CD14, TLR4 and lysozyme. The results shown here indicate that bacteriophages might play a bigger role in the immune response triggered during phage therapy than previously described and might have a broader effect than the clearing of bacterial infections alone, such as the suppression of the immune response.

Introduction

The use of phage therapy has persisted without interruption in Eastern Europe, particularly in centers such as the Eliava Institute of Bacteriophage, Microbiology and Virology in Tbilisi, Georgia and the institute of Immunology and Experimental Therapy in Wroclaw, Poland (Housby and Mann, 2009; Kutter and Sulakvelidze, 2004). Phage interactions with animals in general and human beings in particular have been comprehensively reviewed (Kutter and Sulakvelidze, 2004), and there have been no reports of significant adverse reactions despite their long history of administration to humans.

Viruses and their components are potent activators of signal pathways leading to increased cytokine and chemokine production in human and in animals. The effects exerted on the immune system are usually mediated by viral proteins, which stimulate cytokine and/or reactive oxygen species (ROS) production in immune cells (Thannickal and Fanburg, 2000). Efficacy of phage therapy has been confirmed in various bacterial infections caused by, *e.g.* methicillin-resistant *Staphylococcus aureus* (MRSA) (Capparelli et al., 2007; Mann, 2008; Matsuzaki et al., 2003), *Pseudomonas aeruginosa* (Debarbieux et al., 2010; Watanabe et al., 2007) and *Escherichia coli*, in a number of research centers (Bruttin and Brüssow, 2005; Międzybrodzki et al., 2012). Complementary to the progress in phage therapy practice, advancement of knowledge about the influence of bacteriophages on the mammalian immune system is necessary. Previous studies of bacteriophage interaction with the immune system indicated that at least some phages may exert immunomodulating effects in mammals, such as an important role in transplantation tolerance, the reduction of ROS production, or in the controlling of invading pathogens by adhering of phages on mucosal surfaces (Barr et al., 2013; Górski and Kniotek, 2006; Górski and Weber-Dąbrowska, 2005; Przerwa et al., 2006).

Although some preliminary data on phage-mediated immunobiological activities are available, the exact mechanisms of those interactions remain obscure and require further studies. For example, a study by Ochs *et al.* described the use of phage to study immunodeficiency's in normal and patient populations where high concentrations of phages were administered intravenously without any toxic effects, despite the potent antigenic properties of this particular phage φX174 (Ochs et al., 1971). Humoral antibody responses can be mounted to phages that have been used to immunize animals at high titers (Inchley and Howard, 1969). It has also been shown that phages can inhibit the activation and proliferation of human T cells *in vitro* through an unknown mechanism (Górski et al., 2006b). Since antibodies can decrease phage viability dramatically resulting in the loss of antibacterial effects (Międzybrodzki et al., 2012; Smith et al., 1987), immunogenicity of phages is one of the most important issues that may contribute to the success or failure of therapeutic use of bacterial viruses (Górski et al., 2012; Sulakvelidze et al., 2001). Thus, several studies have shown that phages can trigger a host immune response or can modulate host immunity.

Here we show a transcriptome analysis of human peripheral blood mononuclear cells (PBMCs), derived from one individual, which were stimulated with either *P. aeruginosa* or a *P. aeruginosa* phage PNM lysate, thus containing bacterial proteins but no life active bacteria besides the infectious phage particles. The *P. aeruginosa* phage PNM lysate condition most closely reflects the immunological condition obtained during phage therapy, when the phage titer is the highest and only bacterial fragments remain. These data suggest that certain immunological pathways are activated during/after phage therapy. In addition, it could give a better insight in the efficacy of phages during phage therapy.

Results and discussion

RNA transcriptome analysis

An average of 47 million reads was obtained for the unstimulated and stimulated PBMCs after cleaning and quality checks were carried out. Out of the total number of reads, approximately 85 % (Table 7. 1) could be mapped to the human genome (UCSC version hg19) using the short oligonucleotide analysis package (SOAP) aligner (SOAP2)(Li et al., 2009). The transcriptome analysis from the unstimulated PBMCs versus *P. aeruginosa* strain 573 revealed a total of 996 up-regulated genes and 1377 down-regulated genes compared to the 704 up-regulated and 392 down-regulated genes when the PBMCs were stimulated with *P. aeruginosa* phage PNM.

Comparing both data sets, 359 differentially expressed genes could be identified which were exclusively present in the phage PNM stimulated dataset that did not appear in the *P. aeruginosa* strain 573 dataset. Of these 359 differentially expressed genes, 319 were up-regulated whereas 40 were down-regulated (Addendum -

Table A 2). These genes could be seen as uniquely induced by the phage, although further research is needed.

Gene ontology

Pseudomonas aeruginosa phage PNM

The functional classification based on the biological process revealed that a significant percentage of genes were assigned to immune system response (19.8 %), defense response (13.2 %), response to stress (23.2 %) and regulation of cytokine production (5.3 %). Molecular functions were assigned to, for a high percentage, receptor binding (12.3 %), protein binding (38.2 %), cytokine activity (2.1 %) and cytokine receptor binding (3.2 %). For the functional class of cellular components, many genes were assigned to membrane (56.2 %).

Pseudomonas aeruginosa strain 573

Among the biological process terms, a significant percentage of the genes were assigned to the immune system processes (16.6 %), response to stimulus (34.9 %), defense response (10.5 %) and regulation of cytokine production (4.9 %). Molecular functions were assigned to, for a high percentage, protein binding (36.5) and receptor binding (10.7 %). For the functional class of cellular components, many genes were assigned to membrane (55.9 %), membrane part (47.3 %) and intrinsic to membrane (41.2 %). This might indicate that many genes that are differentially expressed after stimulation with *P. aeruginosa* play some role in signal transduction.

Differential gene expression

Major histocompability complex

Antigens presented on Major Histocompability Complex (MHC) class II are typically exogenous proteins that are endocytosed by the antigen-presenting cell (APC) or endogenous proteins that reside in the secretory system (Schmid et al., 2007, Chapter 2 - Immunology). Analysis of peptides eluted from MHC class II molecules revealed that a substantial proportion of natural MHC class II ligands (up to 20%) are derived from cytosolic and nuclear proteins (Chicz et al., 1993; Dengjel et al., 2005; Dongre et al., 2001).

The major MHC class II genes HLA-DMA, HLA-DMB, HLA-DRB1 and HLA-DRB4 were significantly down-regulated after *P. aeruginosa* phage PNM stimulation. Human HLA-DM plays an important role in the induction and/or stabilization of MHC class II conformation from which weakly bound peptides are able to dissociate. In the absence of HLA-DM, the peptide-editing process fails. This leads to the appearance of weakly bound peptides and peptide-MHC class II conformations that are structurally immature (Watts, 2004).

In case of PBMCs stimulated with *P.* aeruginosa strain 573, our results show that most of the genes involved in the antigen presentation by MHC I and MHC II are

not differentially expressed, with exception of HLA-DRB4 (Log2 fold change of -1.00) and HLA-L (Log2 fold change of 1.02). Gao et al. (2010), using an array approach for porcine PBMCs, reported MHC class II reduction after LPS stimulation.

The CD1c molecule is also significantly down-regulated (P-value = 8.21E-06) in the *P. aeruginosa* phage PNM dataset, the CD1 molecules are MHC-like proteins that bind ß2 microglobulin but, in contrast to MHC class I, their principal domain of action is in the endocytic pathway (Watts, 2004). Two classes of CD1 proteins can be distinguished: those that are recognized by conventional αß T cells (CD1a–CD1c and CD1e) and that present lipid antigens such as mycolic acid and lipopeptides from mycobacteria, and CD1d, which is the restricting element for the specific subset of cells, usually called NKT cells, that express an invariant Vα14 (mouse) or Vα24 (human) T cell receptor and are selected on double-positive thymocytes rather than thymic epithelial cells (Bendelac et al., 1995; Brigl and Brenner, 2004; Joyce and Van Kaer, 2003). By binding to lipid-based molecules, CD1 proteins diversify the range of determinants in foreign pathogens that the immune system can recognize.

These and probably other interactions result in continual cycling of CD1 molecules through the endocytic system but with steady-state distributions that broadly place CD1a and CD1c in early endosomes, and CD1b and CD1d in late endosome and/or lysosomes. Lipid ligand selection is probably controlled by a combination of differential CD1 trafficking and by intrinsic binding-site differences among different CD1 molecules (Gadola et al., 2002; Zajonc et al., 2003).

LPS perception

Lipopolysaccharide perception through TLR4 plays an important role in the immune response against Gram-negative bacteria, such as the bacterial host of phage PNM. We observed a down-regulation of TLR4. Normally TLR4 activation induces the secretion of pro-inflammatory molecules such as chemokines and cytokines which amplify the response to infection (Feezor et al., 2003; Takeuchi et al., 1999).

Both *P. aeruginosa* strain 573 and *P. aeruginosa* phage PNM have the ability to down-regulate TLR4, *i.e.* -3.89 Log2 fold reduction and -1.26 Log2 fold reduction, respectively. In addition *P. aeruginosa* strain 573 was able to down-regulate CD14 (Log2 fold change of -7.96) and lymphocyte antigen 96, encoded by the *LY96* gene (Log2 fold change of -2.04), also known as MD2. Both genes play an important role as co-receptors of TLR4 in the recognition of Gram-negative LPS.

Table 7. 1: Summary of transcriptome analysis of human PBMCs either not stimulated or stimulated with *Pseudomonas aeruginosa* phage PNM or *P. aeruginosa* strain 573.

Stimulation condition	**Unstimulated PBMCs**			**PBMCs stimulated with phage PNM (10^3pfu/PBMC)**			**PBMCs stimulated with *P. aeruginosa* 573 (10^{-1} cfu/PBMC)**		
Biological replicate	a	b	c	a	b	c	a	b	c
Clean reads	46,853,888	46,795,416	47,094,320	47,611,412	47,639,850	47,629,112	47,302,700	48,948,918	47,331,732
Genome map Rate (%)	86.30%	85.80%	85.60%	85.50%	85.60%	85.30%	85.50%	86.10%	85.30%
Gene map Rate (%)	79.30%	79.50%	81.30%	81.00%	80.60%	80.90%	79.20%	78.00%	78.00%
Expressed Genes	16,940	16,968	16,812	16,867	16,885	16,975	17,025	17,099	17,001
Novel Transcripts	644	673	592	683	733	698	746	838	778
Alternative Splicing	55,961	54,175	48,512	59,912	56,444	57,258	58738	60153	59122
SNP	51,195	49,806	46,900	50,326	51,246	51,141	52,877	56,391	54,279

Furthermore, it is interesting to note that lysozyme is strongly down-regulated (Log2 fold change of -7.83) after *P. aeruginosa* strain 573 stimulation. These data suggest that *P. aeruginosa* has evolved a mechanism to circumvent its LPS detection. The precise mechanisms by which the bacteria does this are currently unknown to us.

Pro- and anti-inflammatory signals

Cytokines

Besides the differential expression in the MHC class II, many cytokines were up- and down-regulated. Of the differentially expressed cytokines, the IL10 family cytokines (*i.e.* IL10, IL19, IL20, IL22, IL24, IL26, IL28A and IL28B) are of particular interest for their anti-inflammatory properties and tissue protection. On the one hand, these cytokines prevent excessive tissue damage caused by bacterial and viral infections as well as pro-inflammatory responses. On the other hand, uncontrolled tissue repair processes, such as the wound-healing responses triggered by IL20 subfamily cytokines, can result in diseases, such as psoriasis (Ouyang et al., 2011).

IL10, known for its interaction with leukocytes and its anti-inflammatory properties, had an up-regulation after *P. aeruginosa* phage PNM stimulation (Log2 ratio of 4.32), and after *P. aeruginosa* strain 573 stimulation (Log2 ratio of 4.28). IL10, a T_H2 cytokine that inhibits innate and adaptive immune activities, blocks the expression of pro-inflammatory cytokines, including TNF, IFN-γ, IL1, IL12, and CXC and CC chemokines. The suppression of these cytokines is clearly visible in our data. It also suppresses MHC class II and co-stimulatory molecules CD80 and CD86 on macrophages, and it inhibits the generation of reactive oxygen and nitrogen intermediates from macrophages and neutrophils. Our data indicate a slight up-regulation of CD80 (Log2 ratio of 1.66 for *P. aeruginosa* phage PNM and Log2 ratio of 1.64 for *P. aeruginosa* strain 573) and a down-regulation of CD86 (Log2 ratio of -1.46 for *P. aeruginosa* phage PNM and Log2 ratio of -1.75 for *P. aeruginosa* strain 573).

The IL20 subfamily cytokines, composed of IL19, IL20, IL24 and IL26, primarily acts on various epithelial cells and protects these cells from invasion by extracellular pathogens such as bacteria and yeast. From this group only IL19 (Log2 ratio of 3.1 for *P. aeruginosa* phage PNM and Log2 ratio of 2.77 for *P. aeruginosa* strain 573), IL20 (Log2 ratio of 7.10 for *P. aeruginosa* phage PNM and Log2 ratio of 5.65 for *P. aeruginosa* strain 573) and IL24 (Log2 ratio of 3.22 for *P. aeruginosa* phage PNM and Log2 ratio of 1.1 for *P. aeruginosa*) were differently expressed. In addition, IL20 subfamily cytokines enhance tissue remodeling and wound-healing activities, which help to maintain tissue integrity and restore homeostasis of epithelial layers during infection and inflammatory responses (Ouyang et al., 2011).

IL28A and IL28B, belonging to the type III IFN group were not differentially expressed.

Interleukin-6 (IL6; Log2 ratio of 6.55 for *P. aeruginosa* phage PNM and a Log2 ratio of 6.37 for *P. aeruginosa* strain 573), originally considered to be a pro-inflammatory cytokine, has anti-inflammatory properties (Spooren et al., 2011). For instance, IL6 inhibits neutrophil accumulation after LPS injection and antagonizes the actions of interleukin-1ß (IL1ß) and tumor necrosis factor-α (TNFα) via induction of the soluble IL1 receptor antagonist and the soluble TNFα receptor (Ulich et al., 1991). Under certain conditions IL6 obtains anti-inflammatory characteristics in macrophages (Yasukawa et al., 2003). Finally, IL6 is crucially involved in the induction and regulation of a novel type of T cells, the so-called T_H17 cells (named after the cytokine IL17 which they produce), which are important players in autoimmune reactions. T_H17 cells are believed to be important in autoimmune responses, whereas T_{reg} cells (from T regulatory cells) are suppressors of autoimmune responses and protect against tissue injury. IL6 is crucial for the induction of T_H17 cells (Bettelli et al., 2006; Veldhoen et al., 2006). The inhibitory effect of IL6 and TGFß was suggested to be due to induction of the anti-inflammatory cytokine IL10 and the failure to up-regulate pro-inflammatory chemokines (Spooren et al., 2011).

Of particular interest was the up-regulation of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFα; Log2 ratio of 1.17 and Log2 ratio of 2.77 for *P. aeruginosa* phage PNM and *P. aeruginosa* strain 573 respectively), interleukin 1ß (IL1ß; Log2 ratio of 4.44 for *P. aeruginosa* phage PNM and Log2 fold change of 2.01 for *P. aeruginosa* strain 573), and IL6, characteristic for the classically activated macrophages (M1 phenotype), as well as the up-regulation of anti-inflammatory mediators, such as IL1 decoy receptor (IL1RN) – also known as the IL1 receptor antagonist (IL1ra; Log2 ratio of 3.78 for *P. aeruginosa* phage PNM and Log2 ratio of 2.50 for *P. aeruginosa* strain 573) and IL10, associated with alternative macrophage activation (M2 phenotype) (Goldmann et al., 2004; Gordon, 2003; Mosser, 2003).

Chemokines

The migration of dendritic cells to tissues and from tissues to lymph nodes is central for immune surveillance, priming and tolerance. Monocytes and "immature" dendritic cells exit blood in tissues and scavenge for pathogens. These cells express the "inflammatory" chemokine receptors CCR1 (Log2 ratio of -1.41 for *P. aeruginosa* phage PNM and Log2 ratio of -6.25 *P. aeruginosa* strain 573), CCR2 (Log2 ratio of -3.51 for *P. aeruginosa* phage PNM and Log2 ratio of -3.31 for *P. aeruginosa* strain 573), CCR5 (Log2 ratio of -1.21 for *P. aeruginosa* phage PNM and Log2 ratio of -2.42 for *P. aeruginosa* strain 573), CXCR2 and CCR6 as well as receptors for classical chemo-attractants, formyl peptides and C5a/GPR77 (Log2 ratio of -3.85 for *P. aeruginosa* phage PNM and Log2 ratio of -10.24 for *P. aeruginosa* strain 573) (Cook

et al., 2000; Dieu et al., 1998; Mackay, 2001; Sallusto et al., 1998; Sozzani et al., 1998). One of the ligands of CCR4, CCL17 (thymus- and activation-regulated chemokine, or TARC), is expressed in inflamed skin but not mucosal tissues and is down-regulated by *P. aeruginosa* phage PNM and by *P. aeruginosa* strain 573 (Log2 ratio of -3.26 and -3.06, respectively) (Mackay, 2001).

Chemokines are typically induced in either monocytes/macrophages or in epithelial, endothelial or fibroblastic cells by pro-inflammatory cytokines (*e.g.* IL1 or TNFα) or stimuli (LPS) (Rossi and Zlotnik, 2000). This is in fact the most common perceived role for chemokines, a pro-inflammatory function, frequently associated with a T_H1 cytokine expression profile (IFN-c, IL2, IL12) and thus with a T_H1 cell infiltrate at the inflammation site. However, not all chemokines fit this pattern. For example, other chemokines (*e.g.* C10, DC-CK1/AMAC-1/PARC) are specifically induced by T_H2 cytokines (IL 4, IL10, IL13) in monocytes or other cells (Kodelja et al., 1998; Orlofsky et al., 1994; Rossi and Zlotnik, 2000). Several chemokine receptors are associated with the T_H1 phenotype (including CXCR3 and CCR5), while others are associated with the T_H2 phenotype (CCR3, CCR4, and CCR8) (O'Garra et al., 1998).

Adaptive immunity begins in lymphoid organs, where mature dendritic cells or macrophages present immunogenic peptides to naive or memory T cells. This encounter is governed with remarkable precision by two chemokines, CCL19 (Log2 ratio of 4.33 for *P. aeruginosa* phage PNM and Log 2 ratio of 6.61 for *P. aeruginosa* strain 573) and CCL21, and their receptor, CCR7 (Charo and Ransohoff, 2006; Cyster, 1999, 2003; Sozzani et al., 2000). Once immature dendritic cells ingest antigens and become able to present antigens to T cells, they increase their display of CCR7 (Mantovani, 1999). These $CCR7^+$ dendritic cells enter lymph nodes through the afferent lymph or the bloodstream, using vessel-bound CCL19 and CCL21 to sense their destination. The absence of the up-regulation of CCR7 indicates that *P. aeruginosa* phage PNM is not ingested by the dendritic cell and thus no phage antigens are presented to T-cells, further experiments need to be performed to validate this observation.

Macrophage inflammatory protein 1α (MIP-1α)/CCL3 chemo-attracts a variety of cells, including lymphocytes, monocytes, basophils, and eosinophils (Koch, 2005; Szekanecz et al., 2003). MIP-1α /CCL3, produced by CD44 T cells, is augmented by IL15 (Log2 ratio of 1.08 for *P. aeruginosa* phage PNM and Log2 ratio of 1.16 for *P. aeruginosa* strain 573) stimulation (Wang and Liu, 2003). MIP-3α /CCL20 is a CC chemokine that chemo-attracts T cells, B cells, monocytes, and immature dendritic cells.

P. aeruginosa phage PNM but also its bacterial host is able to reduce the production of CCR1 (Log2 ratio of -1.41 and -6.25, respectively) and CCR2 (Log2 ratio of -3.51 and -3.31, respectively). The up-regulation of CCR1 and CCR2 are clinical indications of rheumatoid arthritis (Charo and Ransohoff, 2006; Katschke et al., 2001; Matsui et al., 2001; Szekanecz et al., 2003). Manipulation of the CCR2

receptor has produced varied outcomes. Antibody-mediated blockade of CCR2 during initiation of collagen-induced arthritis (CIA) in mice resulted in markedly improved clinical signs of arthritis, while blockade during disease progression (therapeutic administration) worsened the clinical and histologic signs of arthritis (Brühl et al., 2004). Elevated levels of CC chemokines, particularly CCL2 (Log2 ratio of -6.57 for *P. aeruginosa* strain 573) and CCL3 (Log2 ratio of 4.52 for *P. aeruginosa* phage PNM and Log2 ratio of 4.01 for *P. aeruginosa* strain 573) in the joints of patients with rheumatoid arthritis coincide with the recruitment of monocytes and T cells into synovial tissues. It appears that targeting CCR1/CCR5 may be useful in experimental arthritis. This indicates that *P. aeruginosa* phage PNM might reduce symptoms associated with rheumatoid arthritis.

It is interesting to note that *P. aeruginosa* phage PNM seems to have the ability to up-regulate the production of superoxide dismutase 2 (SOD2; Log2 ratio of 2.21), which can play an important role in the reduction of ROS, which could lead to a possible explanation of the observation of Miedzybrodzki *et al.* (2008).

Suppressors of cytokine signaling (SOCS)

Suppressors of cytokine signaling (SOCS) proteins are a family of intracellular proteins that control cytokine signaling by suppressing cytokine signal transduction process. In total there are eight members known, SOCS1 to 7 and cytokine-inducible SH2-containing protein (CIS). Studies have shown that SOCS1 and 3 specifically participate in regulation of T_H1 and T_H2 cytokine signaling (Diehl and Rincón, 2002; Dong et al., 2009). SOCS1 (Log2 ratio of 1.87 for *P. aeruginosa* phage PNM and Log2 ratio of 1.40 for *P. aeruginosa* strain 573) is a key modulator of interferon-γ (IFN-γ) signaling. Mice lacking SOCS1 exhibit deregulated responses to IFN-γ resulting in excessive T-cell activation and are hyper-responsive to viral infections (Alexander et al., 1999; Marine et al., 1999). SOCS3 (Log2 ratio of 2.02 for *P. aeruginosa* phage PNM, no differential expression for *P. aeruginosa* strain 573) functions to control IL6 induced T_H2 associated response via its receptor gp130 (Lang et al., 2003). In macrophages, SOCS3 mediates IL10 inhibition of TNFα and nitric oxide production (Qasimi et al., 2006). Induction of SOCS3 is regulated via IL6 receptor trans-signaling. The observation of increased IL10 in SOCS3 over-expressed trophoblast after IL6 challenge is in line with the concept of the pro-inflammatory role of IL6 in trophoblasts and SOCS3 signaling in regulation with cytokine production (Egwuagu et al., 2002).

Both SOCS1 and 3 can inhibit JAK tryrosine kinase activity and can thus interfere with cytokine signaling (Kubo et al., 2003). It has been suggested that SOCS3 negatively regulates IL6, which regulates macrophage activation, B cell development, the production of hepatic acute-phase proteins and the initiation of organ repair (Bernad et al., 1994; Croker et al., 2003; Ramsay et al., 1994). Expression of SOCS3 is induced by numerous factors including Toll-like receptors (TLR) agonists (*e.g.* LPS or CpG-DNA), IL10, IL6 and other gp130/IL6ST signaling

cytokines, leptin and IFN-γ (Cassatella et al., 1999; Lang et al., 2003). The *Socs3* gene has also been shown to be a direct target of STAT3 (Auernhammer et al., 1999). These properties suggest that SOCS3 may broadly regulate cytokine signaling and might be a chief factor in reducing inflammation, particularly in activated macrophages (Lang et al., 2003).

The IL10-mediated induction of SOCS3 in macrophages has led to the notion that SOCS3 is an essential component of the anti-inflammatory effects mediated by IL10 (Berlato et al., 2002). Recent studies have established strong potential for SOCS proteins to regulate M1 and M2 macrophage polarization (Arnold et al., 2014; Qin et al., 2012; Whyte et al., 2011; Wilson, 2014). Overall it is proposed that a high SOCS1 to SOCS3 ratio could be a potential marker for M2 macrophages while high SOCS3 expression is associated with M1 cells (Wilson, 2014). It is clear that SOCS3 is higher expressed than SOCS1, indicating that the immune response induced by *P. aeruginosa* phage PNM is most likely associated with M1 macrophages.

However, it is not only macrophages that are affected by SOCS proteins. Other cell types up-regulate and react to SOCS proteins to shape cellular functions. Targeting SOCS specifically on macrophages is therefore important as an efficient means of changing the inflammatory response (Wilson, 2014).

Conclusion

Our results clearly show that *P. aeruginosa* phage PNM induces an immune response (*e.g.* the expression of TGFBI, IL1 and IL6 after 20 h stimulation of PBMCs), which we further evaluate in **Chapter 8** using highly purified phages. We show that this phage in a phage therapy setting (*i.e.* in the presence of bacterial proteins and membrane fragments) is able to interact with human immune cells and induce an immune response (such as the induction of IL6, IL10, SOD2, SOCS1 and SOCS3).

The group of Gorski and Dąbrowska have recently shown that the T4 phage head surface proteins gp23*, gp24*, Hoc and Soc, both as elements of the phage capsid and as isolated agents, do not induce an IL10 response (Dąbrowska et al., 2014a; Miernikiewicz et al., 2013). Nor did they detect the presence of pro-inflammatory cytokines such as TNFα, IL1 and IL6 in mice, murine dendritic cell cultures or human blood. In contrary to what we show, they postulated (based on their findings) that bacteriophages do not induce an immune response. This difference in results might be explained in two ways, first by the use of different incubation times (where we used 20 h as a stimulation time), and second by the use of different detection platforms (*i.e.* gene expression compared to physiological secretion of cytokines). Although the up-regulation of certain genes does not necessarily mean the secretion of an active protein or the induction of a biological effect, it does indicate the potential of phages to interact with the mammalian immune system.

On the other hand, they did observe the presence of specific T4-antibodies, indicating prior exposure to this phage. This exposure could occur due to the fact that phages are present in food, water and as a natural part of the animal and human gut microbiome (Letarov and Kulikov, 2009).

Recently Liu et al. (2014) showed that phage λ has a physical adaptation to the environment of *E. coli* in a human host. The phage λ genome undergoes a solid-to-fluid-like disordering transition as function of temperature, resulting locally in less densely packed DNA, reducing DNA-DNA repulsions. Once phage λ is inside the host, the increased temperature induces the necessary mobility of the viral genome, facilitating its infection of bacterial cells. This demonstrates an evolutionary physical adaptation of viruses to their host environment (Liu et al., 2014). It can thus be speculated that phages that infect bacteria present in a mammalian host might have numerous adaptations, which leads to their survival and multiplications in a mammalian host.

Although bacteriophages, viruses of bacteria, are mainly known for their relationship with bacteria, they are clearly able to interact with a broader range of eukaryotic cells. It is clear that although phages are known for almost one hundred years, not all is known of these bacterial viruses and further research into this field is warranted.

Material and methods

Culture of Pseudomonas aeruginosa

Pseudomonas aeruginosa strain 573 (received from the Eliava IBMV, Tbilisi, Georgia) was grown on a Lysogeny Broth (LB) agar plate (Becton Dickinson, Erembodegem, Belgium) and incubated overnight at 37 °C. One colony was subsequently used to inoculate a 15 ml tube containing a 4 ml LB agar slant, and incubated overnight at 37 °C. Five ml saline was added to yield a suspension with a final concentration of 10^7 cfu/ml, as confirmed by culture of serial hundredfold dilutions.

Bacteriophage preparation

Phage propagation: Bacteriophage stocks were prepared using the double-agar overlay method as described in Merabishvili *et al*. (2009). Briefly, one ml of phage preparation containing 10^6 plaque forming units (pfu) of bacteriophages was mixed with 3 ml of molten (45 °C) LB top Bacto agar (0.6 %) (Becton Dickinson) and 100 µl of the host strain suspension (end concentration of 10^7 cfu/ml) in a sterile 14 ml tube (Falcon, Becton Dickinson). This mixture was plated onto freshly made 90 mm diameter Petri dishes (Plastiques Gosselin, Menen, Belgium), filled with a bottom layer (20 ml) of 1.5 % LB agar, and incubated aerobically at 32 °C for 16 h. Subsequently, 200 µl of chloroform was added to the lids of the Petri dishes and the inverted plates were further incubated at 4 °C for 1 h. The top layer of the double-

agar layer was scraped off using a sterile Drigalski spatulum and transferred to a sterile 50 ml tube.

The harvested phages were centrifuged for 20 min at 6,000 x *g* at 4 °C. The supernatant was aspirated using a sterile 10 ml syringe (BD Plastipak, Becton Dickinson) with a 30 G sterile needle (BD Microlance 3, Becton Dickinson) and passed through a 0.22 µm membrane filter (Sartorius Stedim, Zellik, Belgium). The filtrate was subsequently centrifuged at 35,000 x *g* for one hour. The phage pellet was resuspended in 5 ml saline and stored at 4 °C overnight before determining the phage titer. Preferably, the titer of the phage lysate should be checked at least one day later according to the above described procedures. This will allow phage particles that may have clumped together during centrifugation steps to disengage (Kutter and Sulakvelidze, 2004).

Phage titer determination: The bacteriophage titer was determined by assaying decinormal serial dilutions (log(0) to log(-12)) of the bacteriophage suspension with the overlay method (Merabishvili et al., 2009). One ml of each dilution was mixed with 3 ml of molten (45 °C) LB 0.6 % top Bacto agar and the host strain (end concentration of 10^7 cfu/ml) in a sterile 14 ml tube. This mixture was plated in triplicate onto 90 mm diameter Petri dishes, filled with a bottom layer of 1.5 % LB agar, and incubated for 16 h at 37 °C. To determine the original bacteriophage concentration, plates with one to 100 distinguishable plaques were counted. The mean was then calculated for the triplicate plates.

PBMC isolation

Peripheral Blood Mononuclear Cells (PBMCs) were isolated from a buffycoat after informed consent (Blood Transfusion Centre, Ghent), using a Lymphoprep (Axis-Shield, Dundee, Scotland) gradient. Fifty ml of the buffycoat was added to 250 ml Hank's Balanced Salt Solution, without Ca^{2+} and Mg^{2+} (HBSS) (Invitrogen). Of this dilution, eight aliquots of 35 ml were each added to 15 ml Lymphoprep in a 50 ml Falcon tube. These mixtures were subsequently centrifuged at 500 x *g* for 20 min at room temperature. The inner whitish ring of PBMCs, present between the lymphoprep and the plasma phase, was transferred to 25 ml HBSS and centrifuged at 450 x *g* for 10 min at room temperature. The supernatants was removed and the cell pellet was resuspended in 10 ml HBSS. All resuspended cells were pooled into a 50 ml Falcon tube and HBSS was added to a total volume of 50 ml. A small fraction of this cell solution was used to count the number of cells present, before it was centrifuged again at 350 x *g* for 10 min at room temperature.

The total number of cells was counted using a Sysmex KX-21 (Sysmex, Norderstedt, Germany). The cell pellet was resuspended in heat-inactivated fetal calf serum with 10 % dimethyl sulfoxide (DMSO) to a concentration of 2 x 10^7 cells/ml and divided in 1 ml aliquots before cryostorage them in liquid nitrogen.

Stimulation of PBMCs

Stimulations were performed in 100 µl volumes containing 10^6 PBMCs. One vial of stored PBMCs, containing 2 x 10^7 cells/ml, was thawed at 37 °C prior to adding 9 ml HBSS (without Ca^{2+} and Mg^{2+}). This suspension was subsequently centrifuged at 350 x *g* for 10 min. The obtained cell pellet was resuspended in 5 ml HBSS (without Ca^{2+} and Mg^{2+}) and 80 µl was used for cell counting on a Sysmex KX-21. This cell suspension was centrifuged at 350 x *g* for 10 min. The resulting cell pellet was resuspended in RPMI 1640 (supplemented with 2 mM L-glutamic acid, 1X MEM non-essential amino acids, 1 mM sodium pyruvate, 60 U of penicillin/ml, 10 mg/ml streptomycin, 2 mM L-glutamine and 10 % heat inactivated fetal calf serum) to a final concentration of 10^7 cells/ml (Moore et al., 1967).

The PBMCs (*i.e.* 10^6 PBMCs/100 µl) were subsequently stimulated with 10 µl of the bacterial host (*i.e. P. aeruginosa* at a concentration of 10^7 cfu/ml or 10^{-1} cfu/PBMC), 10 µl of the phage suspensions at concentrations between 10^{11} pfu/ml, *i.e.*, 10^3 pfu/PBMC. As a negative control, 10 µl saline was added to the cells. The PBMCs were incubated for 20 h at 37 °C in 5 % CO_2. All stimulation experiments and controls were carried out on PBMCs derived from one anonymous donor in triplicate. We confirm that all methods were carried out in accordance to relevant guidelines and regulations and that all experimental protocols were approved by the ethical committee of Ghent University (EC/2017/0558).

Extraction of total nucleic acids: After 20 h of PBMC stimulation, the total cell volume was transferred to 1 ml Qiazol (Qiagen, Valencia, CA) and stored at -80 °C for at least 16 h before extracting the RNA. The total RNA fraction was isolated using a semi-automated procedure of NucliSens EasyMag (Biomérieux, Marcy l'Étoile, France). Briefly, 900 µl EasyMag lysis buffer was added to the 1.1 ml Qiazol solution. This mixture was subsequently transferred to an NucliSens EasyMag cartridge. Finally, 100 µl magnetized silica was added and the cartridge was loaded on the machine (according to the manufacturer). In a final step, the nucleic acids were eluted with 35 µl NucliSens EasyMag elution buffer.

DNase digest: DNase digest was performed immediately after nucleic acid extraction to remove DNA from the sample. Five µl of the nucleic extract was added to one µl of DNase (1 U/µl), one µl 10X DNase reaction buffer (Promega, Mannheim, Germany) and three µl of RNase free H_2O to make up a total volume of 10 µl. This mixture was incubated for 30 min at 37 °C. The DNase digestion was terminated by adding 1 µl of DNase Stop Solution and incubating the mixture at 65 °C for 10 min. DNase digested RNA samples were stored at -80 °C.

cDNA Library preparation and sequencing: cDNA preparation and Illumina HiSeq™ sequencing was performed at the Beijing Genome Institute (BGI) as described by Ren *et al.* (2012). The cDNA libraries were prepared according to the manufacturer's instructions (Illumina, San Diego, CA). Beads coated with oligo(dT) were used to isolate eukaryotic poly(A) mRNA from the total RNA. Purified mRNA was then

fragmented in RNA fragmentation buffer (Ambion, Austin, TX). Using these short fragments as templates, random hexamer-primers (Illumina) were used to synthesize the first-strand cDNA. The second-strand cDNA was synthesized using buffer, dNTPs, RNase H and DNA polymerase I. Short double-stranded cDNA fragments were purified with a QIAquick PCR extraction kit (Qiagen) and eluted with elution buffer for end repair and the addition of a terminal 'A' nucleotide. Next, Illumina sequencing adaptors were ligated to the DNA fragments. DNA fragments of a selected size were gel-purified and amplified by PCR. The amplified library was sequenced on an Illumina HiSeq™ 2000 sequencing machine at BGI, using single-end sequencing with an expected library size of 160 bp and a read length of 90 nt.

Raw read filtering, mapping expression quantification: The images generated by the sequencer were converted into nucleotide sequences by a base-calling pipeline. The raw reads were saved in fastq format, and the dirty raw reads were removed prior to analyzing the data. Dirty raw reads were removed according to the following criteria: reads with sequence adaptors, reads with more than 10 % 'N' bases; low quality reads (*i.e.* the percentage of low quality bases is over 50 % in a read, BGI defined the low quality base to be the base whose sequencing quality is no more than 10). All subsequent analyses were based on clean reads.

The reference sequences used were human genome and transcriptome sequences downloaded from the UCSC website (version hg19). Clean reads were respectively aligned to the reference genome and transcriptome using SOAP2 (Li et al., 2009). No more than three mismatches were allowed in the alignment for each read. Reads that could be uniquely mapped to a gene were used to calculate the expression level. The gene expression level was measured by the number of uniquely mapped reads per kilobase of exon region per million mappable reads (RPKM) (Mortazavi et al., 2008).

Differentially expressed gene (DEG) analysis: Using 'The significance of digital gene expression profiles' (Audic and Claverie, 1997), differentially expressed genes between *P. aeruginosa*-stimulated PBMCs and non-stimulated PBMCs were identified, based on the following criteria: False Discovery Rate (FDR) ≤ 0.001 and an absolute fold change ≥ 2. The data discussed have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE95573 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE95573).

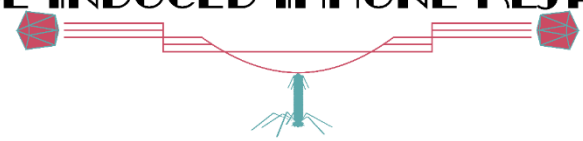
Expression pattern analysis of DEGs: Based on the assumption that genes that have a similar expression pattern usually have a functional correlation, cluster analysis of gene expression patterns was performed using the *cluster* (Langmead et al., 2009) and *javaTreeview* (Saldanha, 2004) software.

Gene ontology (GO) analysis of DEGs: The gene ontology analysis of DEGs was performed by a GO enrichment analysis, which provides all GO terms that are significantly

enriched in a list of DEGs by comparing to a genome background and subsequent filtering the DEGs that correspond to specific biological functions. In this method all DEGs are first mapped to GO terms in the GO database (http://www.geneontology.org), calculating gene numbers for every term, then using hypergeometic tests to find significantly enriched GO terms in the input list of DEGs, based on 'GE::TermFinder' (http://smd.stanford.edu/help/GO-TermFinder/GO_TermFinder_help.shtml).

Chapter 8

Phage Induced Immune Response



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"The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka!' (I found it!), but 'That's funny ...'"

Isaac Asimov (1920 - 1992)

Abstract

The ability of bacteriophages to kill bacteria is well known, as is their potential use as alternatives to antibiotics. As such, bacteriophages reach high doses locally through infection of their bacterial host in the human body. In this study we assessed the gene expression profile of peripheral blood monocytes from six donors for twelve immunity-related genes (*i.e. CD14, CXCL1, CXCL5, IL1A, IL1B, IL1RN, IL6, IL10, LYZ, SOCS3, TGFBI* and *TNFA*) induced by *Staphylococcus aureus* phage ISP and four *Pseudomonas aeruginosa* phages (*i.e*. PNM, LUZ19, 14-1 and GE-vB_Pae-Kakheti25). The phages were able to induce clear and reproducible immune responses. Moreover, the overall immune response was very comparable for all five phages: down-regulation of *LYZ and TGFBI*, and up-regulation of *CXCL1, CXCL5, IL1A, IL1B, IL1RN, IL6, SOCS3* and *TNFA*. The observed immune response was shown to be endotoxin-independent and predominantly anti-inflammatory. Addition of endotoxins to the highly purified phages did not cause an immune response comparable to the one induced by the (endotoxin containing) phage lysate. In addition, the use of an intermediate level of endotoxins tipped the immune response to a more anti-inflammatory response, *i.e.* up-regulation of IL1RN and a strongly reduced expression of CXCL1 and CXCL5.

Importance

We show that bacteriophages are able to induce an (innate) immune response, in contrast to some other recent reports. Additionally, we have strong indications that the phage-induced response is overall anti-inflammatory, which may contribute to the beneficial therapeutic effects of phages during phage therapy.

Introduction

Bacteriophages are the most abundant entities on Earth, impacting ecological niches ranging from the ocean to the gut microbiome (Focà et al., 2015; Hofer, 2013; Mills et al., 2013; Suttle, 2005; Weinbauer, 2004). Furthermore, it is known that phages are immensely specific towards a specific bacterial host without infecting other bacterial strains. Moreover, there is currently no data available on phages infecting eukaryotic cells. This makes them ideal candidates to treat bacterial infections, while being harmless to mammalian cells and even non-target bacteria (Thiel, 2004).

Bacteria that inhabit the intestine and skin are generally regarded as stable residents that may confer metabolic and/or immune benefits to their hosts (Turnbaugh et al., 2009). The host immune system has evolved mechanisms to tolerate these commensal organisms while at the same time providing protection for the host from pathogens (Moon and Stappenbeck, 2012). Similarly, metagenomic studies have revealed that a vast variety of bacteriophages are associated with healthy human tissues (Minot et al., 2011; Pride et al., 2012; Reyes et al., 2010). In

case of phages, a persistent nonpathogenic association seems possible as viral replication occurs only in bacterial hosts, which can themselves be stable members of the microbiome (Duerkop and Hooper, 2013).

It has been demonstrated that oral uptake of phages by animals results in the translocation of phages to systemic tissues (Duerr et al., 2004; Hamzeh-Mivehroud et al., 2008). This suggests that mammals have mechanisms for the uptake and delivery of phages from the gut to the blood. The contact between systemic tissues and phages may allow intestinal phages to elicit innate and adaptive immune responses. One possible uptake route involves dendritic cells, which are known to sample intestinal luminal contents and can actively phagocytize phage particles in culture (Barfoot et al., 1989; Rescigno et al., 2001). It is also interesting to consider whether phages might elicit antiviral innate immune responses. Mammalian cells are endowed with the ability to detect viral nucleic acids through several pattern-recognition receptors that are positioned to detect viral entry into cells (Duerkop and Hooper, 2013).

A study performed by Weber-Dąbrowska *et al.* (2000) demonstrated that phage therapy can normalize TNFα serum levels and the production of TNFα and IL6 by blood cell cultures. Miernikiewicz *et al.* (2013) performed an extensive study of the immunological effects of phage T4 and its head surface proteins. They found that phage T4 and its surface proteins gp23*, gp24*, Hoc and Soc did not affect production of the inflammatory cytokine and ROS production. Recently Majewska *et al.* (2015) followed the antibody production (*i.e.* IgM, IgG and secretory IgA) after oral application of phage T4 to mice. However, the orally applied phage T4 induced anti-phage antibodies only after a combination of long exposure times (*i.e.* IgG day 36 and IgA day 79) and high doses.

The use of phage therapy has persisted without interruption in Eastern Europe, particularly in centers such as the Eliava Institute of Bacteriophage, Microbiology and Virology in Tbilisi, Georgia and the Institute of Immunology and Experimental Therapy in Wroclaw, Poland (Housby and Mann, 2009; Kutter and Sulakvelidze, 2004). Phage interactions with animals in general and human beings in particular have been comprehensively reviewed (Kutter and Sulakvelidze, 2004), and there have been no reports of significant adverse reactions despite their long history of administration to humans.

Here, we report on the immune response induced by individual (highly purified) phages. It has previously been described that CsCl gradient ultra-centrifuged phages are free from residual DNA, RNA and bacterial proteins released during the lysis of the bacterial cell (Reddy et al., 1988). Hence, the immune responses observed in our study are induced by the phages. We previously conducted a whole transcriptome analysis of human peripheral blood mononuclear cells (PBMCs) stimulated with either *P. aeruginosa* or a *P. aeruginosa* phage PNM lysate (Chapter 7). The *P. aeruginosa* phage PNM lysate condition most closely reflects the immunological condition obtained during phage therapy, when the phage titer is

the highest and predominantly bacterial fragments, from the lysed bacterial cells, remain. These data suggest that certain immunological pathways are activated during and/or after phage therapy and may contribute to the efficacy of phages during phage therapy. Next, to understand to what extent the phage particles interact with the human immune system, the immune response induced by two different phage purification strategies (*i.e.* either a phage lysate or a highly purified phage) was compared. Using twelve immunity-related genes, the immune response induced by five different phages were compared, four infecting *P. aeruginosa* and one *S. aureus* phage. These five phages represent the three major phage morphologies (Table 8. 1). This might give us an idea whether the immune response might be phage host specific or phage morphology dependent.

Results

RT-qPCR validation of 12 immunity-related genes.

Staphylococcus aureus phage ISP induced immune response

Stimulation of PBMCs, derived from six donors (each stimulated in triplicate), with either an *S. aureus* phage ISP lysate, the highly purified phage ISP (undiluted and 100-fold diluted), or the *S. aureus* host strain showed that the immune response within each condition was highly reproducible (Figure 8. 1). Diluting the highly purified phage ISP led to an almost complete reduction of the observed immune response, with the exception of TNFA and CXCL5 which have the same induction as the undiluted phage and IL1B and CXCL1 which are both up-regulated but are significantly different from the undiluted phage condition, i.e. up-regulated but significantly less strong than the undiluted phage. These clear titration effects indicate that sufficiently high phage titers (*i.e.* $\geq 10^3$ pfu/PBMC) are necessary in order to induce an immune response.

Interestingly, the highly purified phage ISP induced an almost identical response as its bacterial host strain (except for *CXCL5*, *IL1RN, IL6 and IL10*), whereas the phage lysate, still containing bacterial contamination, induced a much weaker overall response (except for *CXCL1, CXCL5, IL1B, IL1RN* and *TNFA*). The highly purified phage ISP significantly (p-value < 0.05) up-regulates the *CXCL1*, *IL1A, IL1B, IL1RN, IL6* and *TNFA* genes and down-regulates *CD14, LYZ* and *TGFBI* compared to the phage lysate.

The immune response induced by the phage and its bacterial host (*i.e. S. aureus* strain 6538) also shows a strong down-regulation of the *LYZ* gene expression, in agreement with the transcriptome results (Figure 8. 1). The values between the transcriptome analyses and RT-qPCR were consistent ($R^2 = 0.88$).

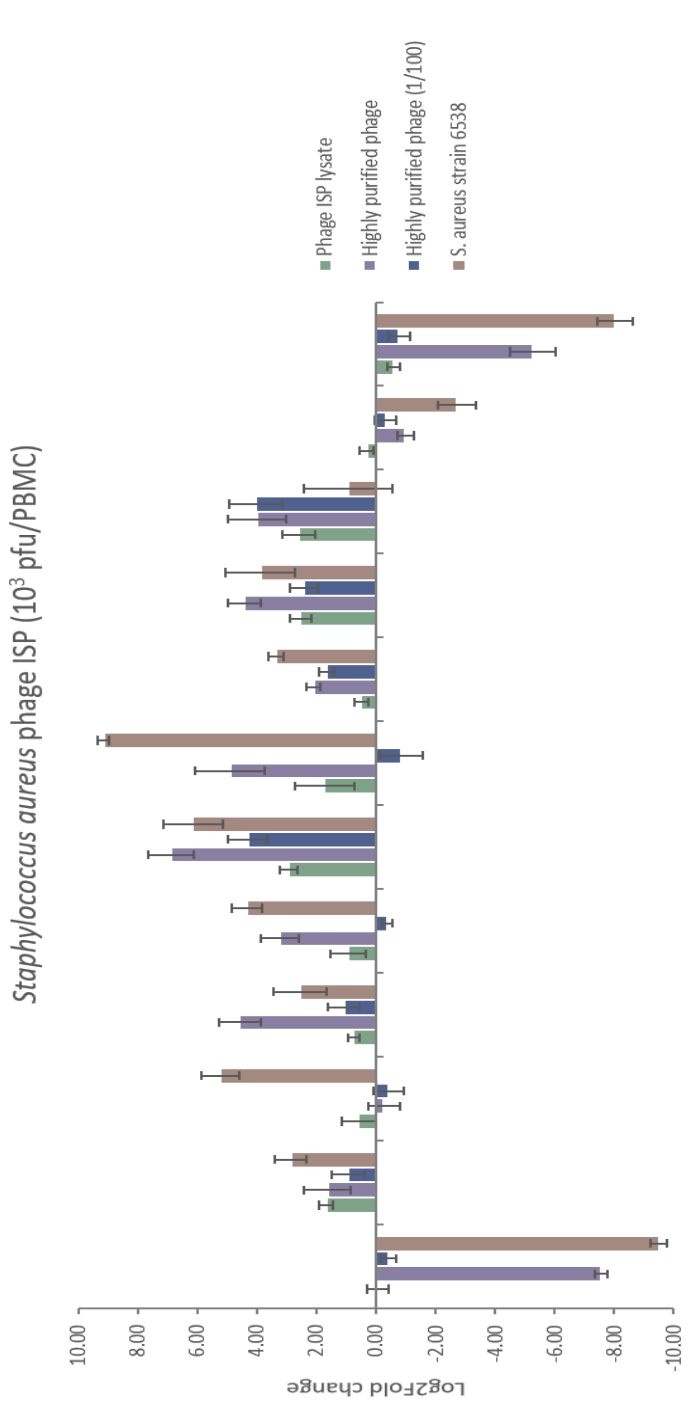


Figure 8. 1: Gene expression analysis of 12 immunity-related genes, assessed by means of RT-qPCR, after 20 h stimulation of PBMCs with *Staphylococcus aureus* strain ATCC 6538 or *S. aureus* phage ISP. Comparison of the immune response of the PBMCs, expressed as log2 fold change, after normalization with reference gene ACTB, as induced by the phage lysate, highly purified phage (undiluted and diluted hundredfold) or its bacterial host (*i.e. S.* aureus strain ATCC 6538). The immune response induced by the highly purified phage preparation is stronger than the one induced by the phage lysate. Diluting of the highly purified phage led to an almost complete reduction of the observed immune response, with the exception of *CXCL5* and *IL1B*. A strong difference is present between the immune response induced by the phage compared to its bacterial host (*i.e. S. aureus* strain 6538).

Influence of endotoxins on the phage-induced immune response

To determine whether the observed immune response was induced by the bacteriophage rather than by possible endotoxin contamination present in the phage lysate or purified phage (Table 8. 1), PBMCs were stimulated with either *S. aureus* phage ISP alone, *i.e.* completely endotoxin free (Table 8. 1), at concentrations of 10^3 pfu/PBMC, or in combination with different concentrations of a commercial endotoxin preparation (*i.e.* 10^{-1}, 10^{-3} or 10^{-7} EU/ PBMC).

The addition of up to 10^{-1} EU/PBMC to 10^3 pfu/PBMC of *S. aureus* phage ISP did not lead to a significant difference in the immune response as induced by the phage alone (Figure 8. 2 A - C). However, the immune response induced by phage ISP at 10^3 pfu/PBMC (with or without endotoxins) differed significantly (p-value < 0.05) from the immune response induced by the endotoxins alone, for *CXCL5, LYZ, TGFBI* and *TNFA*. The combination of phage with endotoxins led to a stronger up-regulation of *TNFA* or stronger down-regulation of *LYZ* and *TGFBI* in comparison with endotoxins alone, whereas the expression of *CXCL5* was reduced compared to endotoxin alone when a combination of phage ISP with endotoxins was used.

Lowering the endotoxin concentration added to phage ISP from 10^{-1} EU/PBMC to 10^{-3} EU/PBMC, resulted in a significant difference (p-value <0.05) in the gene expression of *CXCL1, CXCL5, IL1RN, LYZ, TNFA* and *TGFBI,* between the phage ISP with endotoxins (*i.e.* 10^3 pfu and 10^{-3} EU/PBMC) and endotoxins alone (*i.e.* 10^{-3} EU/PBMC). Of particular interest was the down-regulation of *CXCL1* and *CXCL5* when stimulation was carried out with both endotoxin and phage compared to 10^3 pfu of phage ISP/PBMC alone or 10^{-3} of EU/PBMC alone (Figure 8. 2B). Further reduction of the endotoxin concentration to 10^{-7} EU/PBMC did not significantly differ from the immune response induced by the phage alone, with the exception of *CXCL1* and *IL1A* (Figure 8. 2C), which were less expressed when the PBMCs were challenged with 10^{-7} EU/PMBC added to the phage instead of when stimulated by the phage alone.

Lowering the phage concentration from 10^3 pfu/PBMC to 10 pfu/PBMC, in combination with 10^{-1}, 10^{-3} or 10^{-7} EU/PBMC led to an immune response more similar to the one induced by the endotoxin alone than the one induced by the phage alone (Figure 8. 2D – F).

Pseudomonas aeruginosa phage PNM induced immune response

Comparable to the observations for *S. aureus* phage ISP, the immune response induced by the *P. aeruginosa* phage PNM lysate (at 10^5 pfu/PBMC) differs significantly (p-value < 0.05) from the one induced by the highly purified phage (at 10^5 pfu/PBMC). However, it was the *P. aeruginosa* phage PNM lysate-induced response that was most consistent with the response to its bacterial host strain (10^{-1} cfu/PBMC), except for *CD14*, *CXCL5*, *LYZ* and *TNFA*. A significant difference in the induced immune response between the phage lysate and the purified phage could be observed for *CXCL1*, *IL1A, IL1B, IL6, TNFA* and *TGFBI* (Figure 8. 3).

Because this difference could be due to endotoxin contamination, we compared the stimulation of the PBMCs with a highly purified phage PNM preparation with the addition of endotoxins (*i.e.* 10^{-1} EU/PBMC), in order to equalize the endotoxin concentration with that of the phage lysate (3 x 10^{-1} EU/PBMC, Table 8. 1). Nonetheless, this did not bring the observed immune response closer to the one induced by the phage lysate and even had the opposite effect for *e.g. IL1A*, *IL6* and *IL10* (Figure 8. 3). This indicates that the difference in immune response induced by the highly purified phage compared to the phage lysate is not due to the presence of endotoxins in the phage lysate, but rather due to the presence of bacterial contaminants (*e.g.* bacterial DNA or proteins) in the phage lysate.

Immune response induced by *Pseudomonas aeruginosa* phages PNM, LUZ19, 14-1 and GE_vB_Pae-Kakheti25

All four *P. aeruginosa* phages [*i.e.* PNM (10^5 pfu/PBMC), LUZ19 (10^5 – 10^3 pfu/PBMC), 14-1 (10^4 – 10^3 pfu/PBMC and Ge_vB_Pae-Kakheti25 (10^4 pfu/PBMC)] induced a comparable immune response (Figure 8. 4). For all four phages, there is a clear difference in the level of the gene expression induced in the PBMCs by the stimulation of the phage lysate or the highly purified phage. For all four phages, the phage lysate induces a stronger immune response compared to the highly purified phage.

All four *P. aeruginosa* phages induce *IL6* and the anti-inflammatory genes *IL1RN, IL10* and *SOCS3,* as well as the pro-inflammatory genes *CXCL1, CXCL5, IL1A* and *IL1B*. Only the expression of *TNFA* is different between the four phages, with phage PNM and 14-1 inducing a significant slight up-regulation while phages LUZ19 and GE_vB_Pae-Kakheti25 induced a down-regulation (Figure 8. 4). Moreover, the phage lysates strongly resembled the immune response induced by their bacterial host, *i.e. P. aeruginosa* strain 573.

Discussion

Based on the transcriptome analysis described in Chapter 7 – Transcriptome analysis of phage stimulated PBMCs we were able to show that *P. aeruginosa* phage PNM does activate immunological pathways. Subsequent in depth analysis of twelve selected immunity-related genes (Table 8. 3) by means of RT-qPCR confirmed that five different bacteriophages, one with a Gram-positive host and four with a Gram-negative host (Table 8. 1), were able to induce an immune response. Our study addressed only the cytokine gene expression level and not the cytokine protein level, for several reasons. First, stimulation times for cytokine protein levels are much longer (Kranzer et al., 2004; Zelante and Ricciardi-Castagnoli, 2012) and such longer stimulation times are not optimal for freshly isolated PBMCs. Furthermore, an early response and fluctuations in the response are more rapidly detected at the gene transcription level than at changes in protein concentration.

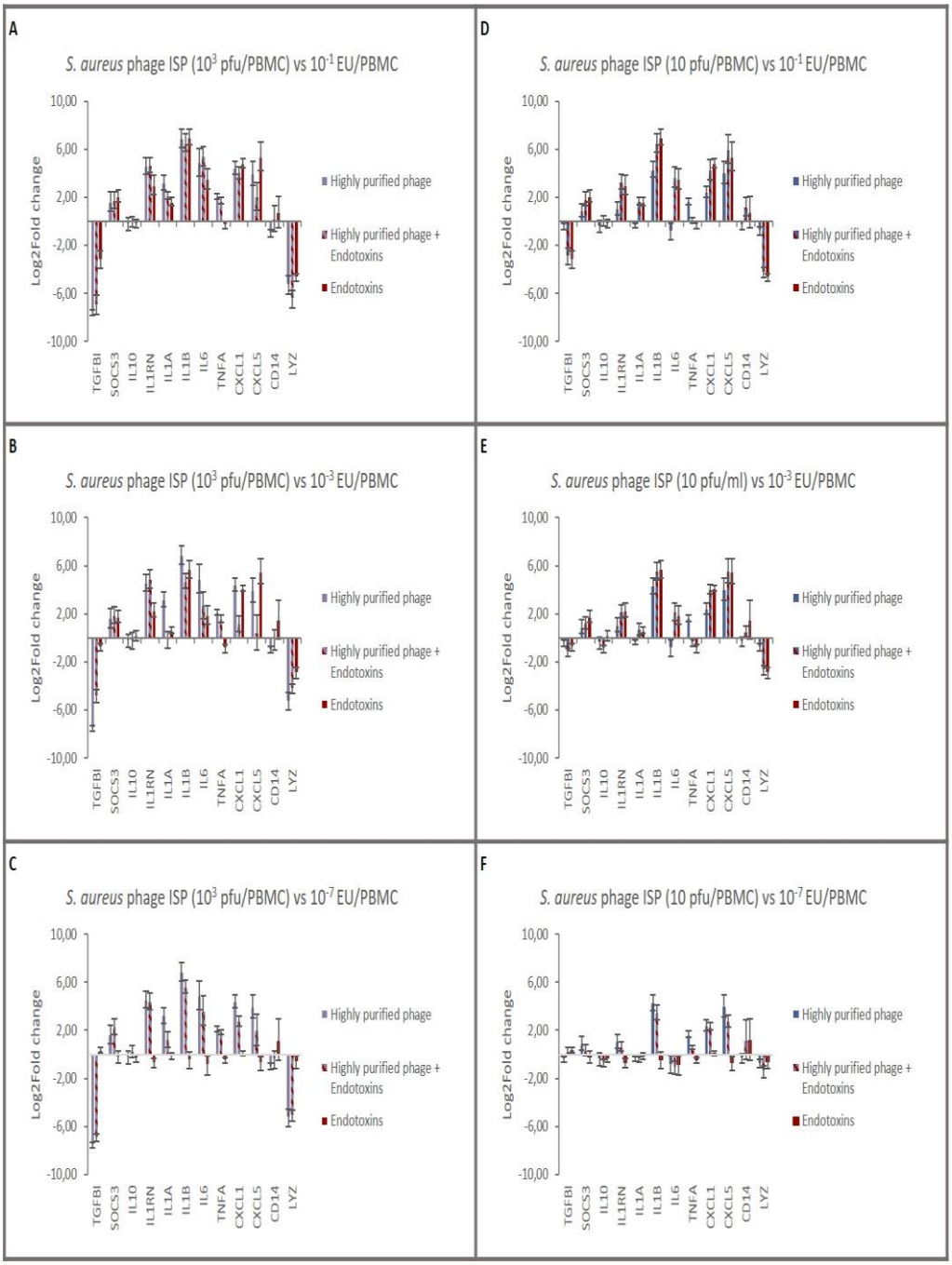

A
S. aureus phage ISP (10³ pfu/PBMC) vs 10⁻¹ EU/PBMC
D
S. aureus phage ISP (10 pfu/PBMC) vs 10⁻¹ EU/PBMC
B
S. aureus phage ISP (10³ pfu/PBMC) vs 10⁻³ EU/PBMC
E
S. aureus phage ISP (10 pfu/ml) vs 10⁻³ EU/PBMC
C
S. aureus phage ISP (10³ pfu/PBMC) vs 10⁻⁷ EU/PBMC
F
S. aureus phage ISP (10 pfu/PBMC) vs 10⁻⁷ EU/PBMC
Log2Fold change
10,00
6,00
2,00
-2,00
-6,00
-10,00
TGFBI
SOCS3
IL10
IL1RN
IL1A
IL1B
IL6
TNFA
CXCL1
CXCL5
CD14
LYZ
Highly purified phage
Highly purified phage + Endotoxins
Endotoxins

Figure 8. 2: Gene expression analysis of 12 immunity-related genes, assessed by means of RT-qPCR, after 20 h of stimulation of PBMCs with *Staphylococcus aureus* phage ISP in combination with endotoxins (*i.e.* 10^{-1}, 10^{-3} or 10^{-7} EU/ml). (A, B, C) 10^3 pfu/PBMC in combination with (A) 10^{-1} EU/PBMC, (B) 10^{-3} EU/PBMC or (C) 10^{--7} EU/PBMC leads to an immune response more similar to the one induced by the phage alone. (D, E, F) Reducing the phage titer to 10 pfu/PBMC causes the immune response to tilt in the direction of the endotoxin-induced immune response instead of the phage induced immune response.

We found that for all five phages, the overall immune response (as determined by RT-qPCR) is very comparable (Figure 8. 5), but differs from the immune response induced by their bacterial host. The observed immune response is in large correspondence with the transcriptome analysis. Moreover, the immune response induced by a large number of phages (*i.e.* 10^3 pfu/PBMC) seems to be endotoxin independent, the addition of endotoxins to the highly purified *S. aureus* phage ISP did not subvert the immune response to the one induced by the *S. aureus* phage ISP lysate. Furthermore, the stimulation of PBMCs with either a *P. aeruginosa* phage PNM lysate or a highly purified *P. aeruginosa* phage PNM preparation supplemented with endotoxins in concentration of 10^{-1} EU/PBMC (Figure 8. 3) did not bring the observed immune response closer to the one induced by the phage PNM lysate, indicating that the immune response induced by the phage preparations is not due to the presence of endotoxins but more due to presence of other bacterial components in the phage lysate preparation. Additionally, it has recently been shown that *Escherichia coli* phage T4 gp12 can decrease the immune response induced by endotoxins, through the binding of endotoxins to gp12 (Miernikiewicz et al., 2016).

Whether phages induce a highly comparable overall immune response or whether only specific parts of the immune response are similar needs to be further evaluated, as we specifically looked at twelve immunity-related genes. However, the ability of these five phages to induce a, largely, similar immune response might be due to the modular nature of phage genomes (Bérard et al., 2016). Based on the modular nature of phages genomes, it can be hypothesized that phage capsid proteins might have similar folds and subsequently induce similar immune responses. For instance, it has been shown that gp23 and gp24 of *E. coli* phage T4 have a similar fold as that of the *E. coli* phage HK97 capsid protein (Fokine and Rossmann, 2014; Fokine et al., 2005). Yet a single point mutation can change the serotype of the phage. This might also be the case for *P. aeruginosa* phage PNM and LUZ19, which are homologous, yet slightly differ in the induced immune response.

Table 8. 1: Different phages used in the PBMC stimulation assay. For each phage, taxonomical family and bacterial host strain are presented, as well as the titer of the phage lysates, before and after endotoxin removal.

Bacteriophage name	Phage family	Bacterial Host	Titer (pfu/ml)	Reference	Isolated by	Isolation date
P. aeruginosa phage PNM	*Podoviridae*	*P. aeruginosa* strain 573	1.8×10^{13}	Merabishvili et al. (2009)	N. Lashki & M. Tediashvili	1999
P. aeruginosa phage LUZ19	*Podoviridae*	*P. aeruginosa* strain 573	5.0×10^{13}	Lammens et al. (2009)	P.J. Ceyssens	2006
P. aeruginosa phage GE-vB_Pae-Kakheti25	*Siphoviridae*	*P. aeruginosa* strain 573	2.5×10^{12}	Karumidze et al. (2012)	N. Kvatadze	2012
P. aeruginosa phage 14-1	*Myoviridae*	*P. aeruginosa* strain 573	3.6×10^{12}	Ceyssens et al. (2009)	V. Krylov	2000
S. aureus phage ISP	*Myoviridae*	*S. aureus* strain ATCC 6538	8.0×10^{13}	Vandersteegen et al. (2011)	Unknown	1920 - 1930

At this point, it is difficult to establish whether the phages are predominantly pro- or anti-inflammatory, although the phages have the tendency to induce an anti-inflammatory response, as assessed based on these twelve immunity-related genes and on the premise of a sufficiently high phage concentration (*i.e.* 10^3 pfu/PBMC). It is on the other hand very clear that they do activate several immunological pathways (Figure 8. 6).

The influence of phages on the cytokine mRNA production, as observed in this study, may not be all that unexpected, since there are several indications for other interactions of phages with human cells. For example, they may play a significant role in transplantation as it was shown that in mice they reduce cellular infiltration of allogenic skin allografts (Górski et al., 2006b). Phages might do this by inhibiting the adhesion of platelets and, to some extent, of T- cells to fibrinogen, a protein which plays an important role in transplant rejection, angiogenesis and metastasis (Gorski et al., 2003; Kurzępa et al., 2009). A more recent study demonstrated that bacteriophages are also able to inhibit the production of reactive oxygen species (ROS) as part of the response of granulocytes to the presence of bacteria, which may be beneficial because overproduction of ROS leads to tissue damage (Miedzybrodzki et al., 2008; Przerwa et al., 2006). Another study proved that phages can aid in the killing of phagocytosed *S. aureus* (Kaur et al., 2014). The authors showed that phage particles absorbed to the bacterial surface have an impact on the killing of engulfed *S. aureus* inside phagocytic cells (*e.g.* macrophages) by 38.7 %. Furthermore, it has been indicated that phages are able to interact with human mucosal surfaces and from a non-host derived immune barrier (Barr et al., 2013, 2015).

It was recently shown that the surface proteins gp23*, gp24*, Hoc and Soc of phage T4 do not induce an *IL10* response, neither as elements of the integral phage capsid nor as isolated proteins. This is in accordance to our findings for *S. aureus* phage ISP, but not for the *P. aeruginosa* phages (Dąbrowska et al., 2014a; Miernikiewicz et al., 2013). Nor did these authors detect the presence of pro-inflammatory cytokines such as TNFA, IL1 and IL6 in mice, murine dendritic cell cultures or human blood. Contrary to what we show here, they postulate (based on their findings) that bacteriophages do not induce an immune response. This difference in results might be explained by the use of different incubation times, since we used 20 h as a stimulation time (optimal time frame for the detection of *IL10* mRNA) instead of 5 – 6 h. Another major difference is that they studied an *Escherichia coli* phage (T4), which might suggest a species/phage specific response.

The up-regulation of *IL1RN* by all five phages, as shown in our study, is of particular interest. All five phages induce the pro-inflammatory cytokines IL1A and IL1B, but the simultaneous induction of the anti-inflammatory IL1 receptor antagonist (IL1RN) will interfere with the pro-inflammatory IL1 signal and thus dampen the IL1 pro-inflammatory response.

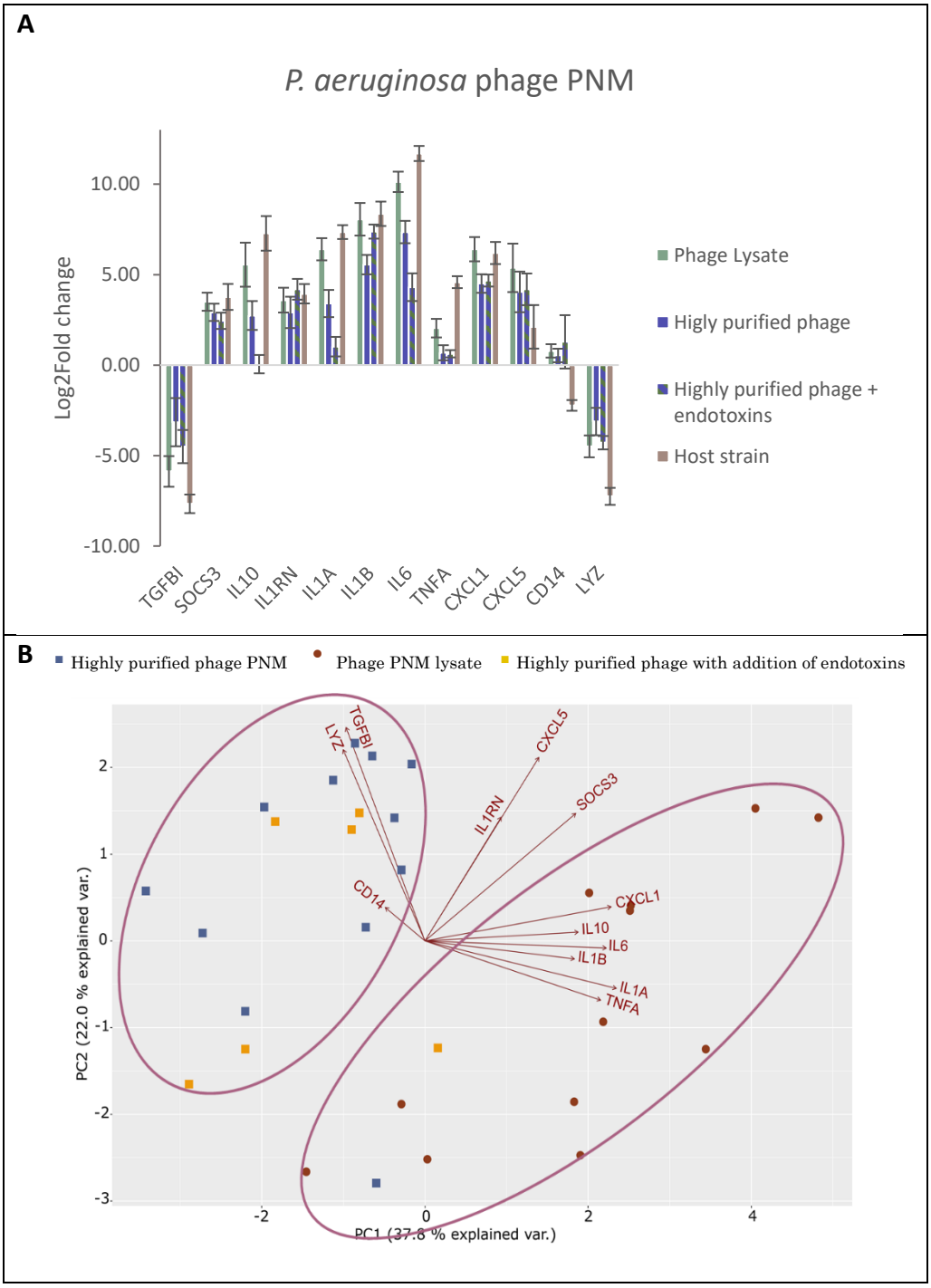

A
P. aeruginosa phage PNM
Log2Fold change
10.00
5.00
0.00
-5.00
-10.00
TGFBI
SOCS3
IL10
IL1RN
IL1A
IL1B
IL6
TNFA
CXCL1
CXCL5
CD14
LYZ
Phage Lysate
Higly purified phage
Highly purified phage + endotoxins
Host strain
B
Highly purified phage PNM
Phage PNM lysate
Highly purified phage with addition of endotoxins
PC2 (22.0 % explained var.)
PC1 (37.8 % explained var.)
TGFBI
LYZ
CXCL5
IL1RN
SOCS3
CD14
CXCL1
IL10
IL6
IL1B
IL1A
TNFA

Figure 8. 3: Effect of endotoxins in the presence *P. aeruginosa* phage PNM. A| Gene expression analysis of 12 immunity-related genes by means of RT-qPCR after 20 h of stimulation of PBMCs. PBMCs were stimulated with *P. aeruginosa* phage PNM, either a phage lysate (10^5 pfu/PBMC; 0.1 EU/PBMC) or a highly purified phage preparation (10^5 pfu/PBMC; 10^{-5} EU/PBMC) in combination with 0.1 EU/PBMC. The pro-inflammatory markers *IL1A, IL1B, CXCL1* and *CXCL5*, and the anti-inflammatory markers SOCS3, IL10, *IL1RN* and *IL6* are upregulated The addition of 0.1 EU/PBMC to the highly purified *P. aeruginosa* phage preparation does not revert the observed immune response to that of the phage lysate for *IL10, IL1A, IL6, TNFA* and *CXCL1*. **B| Principal components analysis of *P. aeruginosa* phage PNM with or without the addition of endotoxins.** The immune response induced by the highly purified phage PNM (▪) differs from the one induced by the phage PNM lysate (•), as these two groups are visibly separated. When endotoxins are added to a final concentration of 0.1 EU/PBMC to the highly purified phage (▪), the immune response is similar to the highly purified phage (▪) and not towards the phage lysate (•), indicating that the observed difference is not due to the presence of LPS but due to bacterial proteins present in the phage lysate

In a previous study, Chapter 6 – Endotoxin purification strategies, we showed the difficulty to remove endotoxins from phage preparations (Van Belleghem et al., 2017a), making it impossible to completely remove all endotoxins from high titer *P. aeruginosa* phage preparations. To exclude that the observed immune response for the *P. aeruginosa* phages were, partially, due to incomplete endotoxin removal, we compared the response of *S. aureus* phage ISP, shown to be completely endotoxin-free with that of ISP phage in combination with three concentrations of endotoxins (10^{-1}, 10^{-3} or 10^{-7} EU/PBMC). Our results strongly indicate that the addition of even the highest concentration of endotoxins to a high concentration of *S. aureus* phage ISP (10^3 pfu/PBMC) has no effect on the induced immune response (Figure 8. 2A). Intriguingly, when an intermediate level of endotoxins was added, the immune response tipped to a more anti-inflammatory response (*i.e.* an up-regulation of IL1RN and a strongly reduced expression of CXCL1 and CXCL5) and differed from that after stimulation with the highly purified phage or with only endotoxin.

Conclusions

The up-regulation of *IL1RN* (Interleukin-1 Receptor Antagonist) and *SOCS3* (Suppressor of Cytokine Signaling 3) by all five phages, or the down-regulation of *CXCL1* and *CXCL5* by *S. aureus* phage ISP in addition of endotoxins, might indicate that these phages have evolved anti-inflammatory properties while some pro-inflammatory properties remain (*e.g.* up-regulation of *IL1A, IL1B* and *TNFA*).

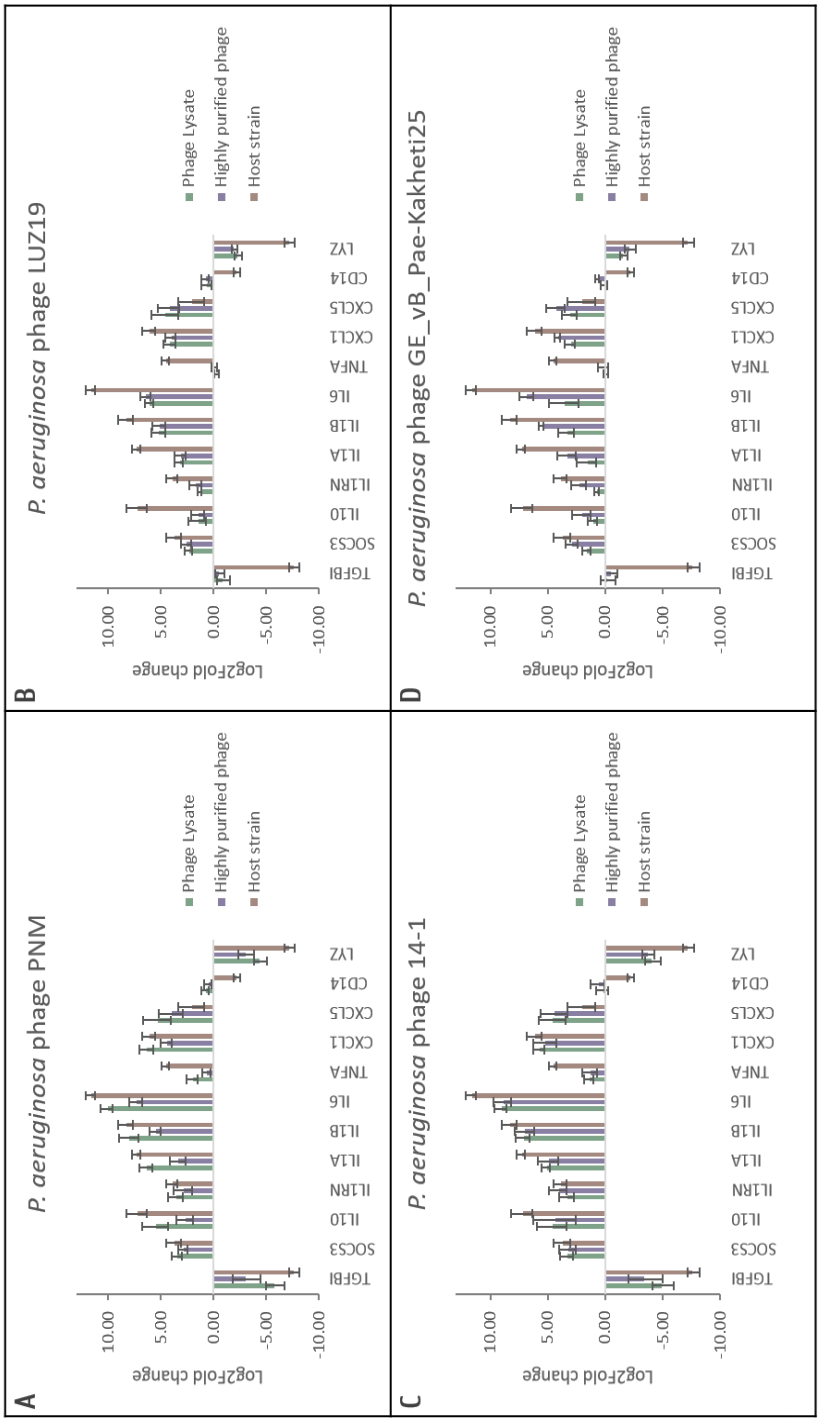


Figure 8. 4: Gene expression analysis of 12 immunity-related genes by means of RT-qPCR after 20h stimulation of PBMCs. PBMCs were stimulated with either (A) *P. aeruginosa* phage PNM, (B) *P. aeruginosa* phage LUZ19, (C) *P. aeruginosa* phage 14-1 or (D) *P. aeruginosa* phage GE_vB_Pae-Kakheti25. The pro-inflammatory markers *IL1A*, *IL1B*, *CXCL1* and *CXCL5* and the anti-inflammatory markers *SOCS3*, *IL10*, *IL1RN* and *IL6* are up-regulated. Only phages PNM (A) and 14-1 (C) slightly up-regulate *TNFA*, whereas phages LUZ19 (B) and GE_vB_Pae-Kakheti25 (D) cause a slight down-regulation of *TNFA*.

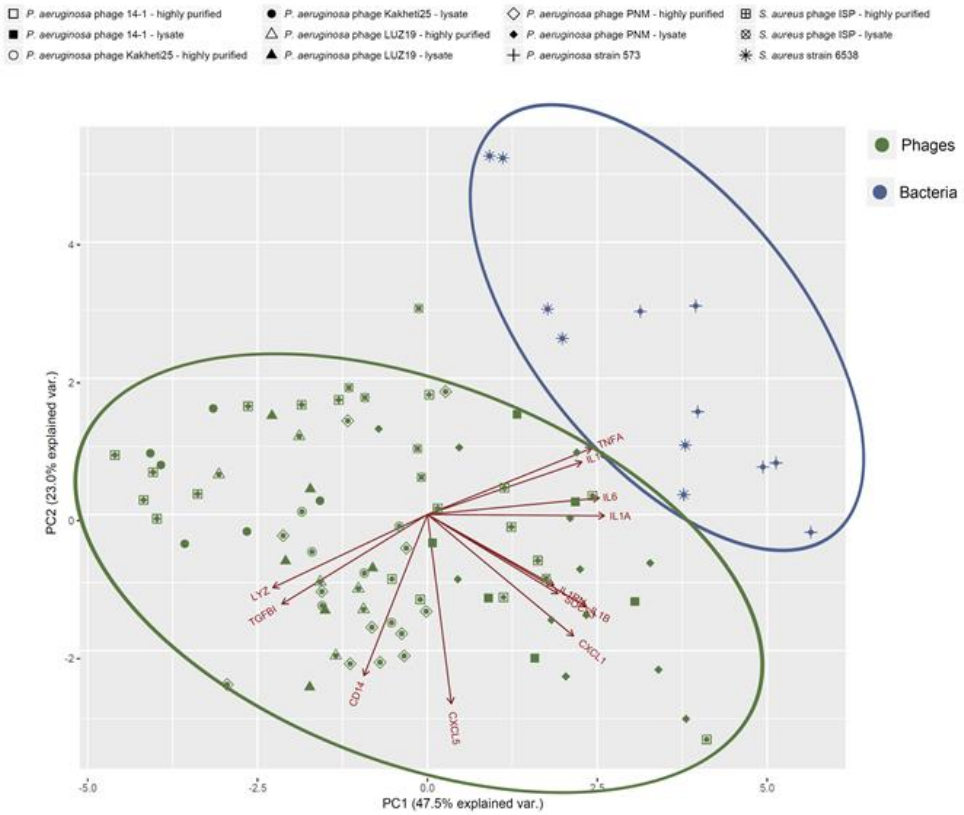


Figure 8. 5: Principal component analysis (PCA). Comparison of the immune response of PBMCs induced by all five tested phages (Highly purified or phage lysate; magenta) and their bacterial host strain (blue). The PCA clearly shows that the bacteria (blue) and phages (magenta) for two separate groups, indicating that the immune response induced is different between these two types of stimuli. There is no clear distinction between the different phages, indicating that these five different phages induced similar responses. Moreover the gene expression of *IL1RN, IL1B* and *SOCS3, IL6* and *IL1A*, *TNFA* and *IL10,* and *LYZ* and *TGFBI* are correlated

It could thus be theoretically postulated that by reducing the immune response, phages first reduce the chance to be removed from the human body and be degraded. Secondly, these responses might promote the survival of their bacterial host which in turn provides the phage with a higher fitness and better opportunities to proliferate but also to remove the pathogenic bacteria, in case of bacterial infections. This could be further argued from the point of view that when the phage concentration reaches a certain threshold this could lead to lysis from without. When the phage subsequently reduces the immune response and the bacterial

concentration rises, the overall phage to bacterial concentration could drop below the lysis from without threshold enabling the phages to once again infect the bacteria and propagate. With regard to phage therapy, these anti-inflammatory effects of phages might increase the capacity of the phages to suppress bacterial numbers and thus infections, with the addition of dampening the negative site of the inflammatory response.

Material and methods

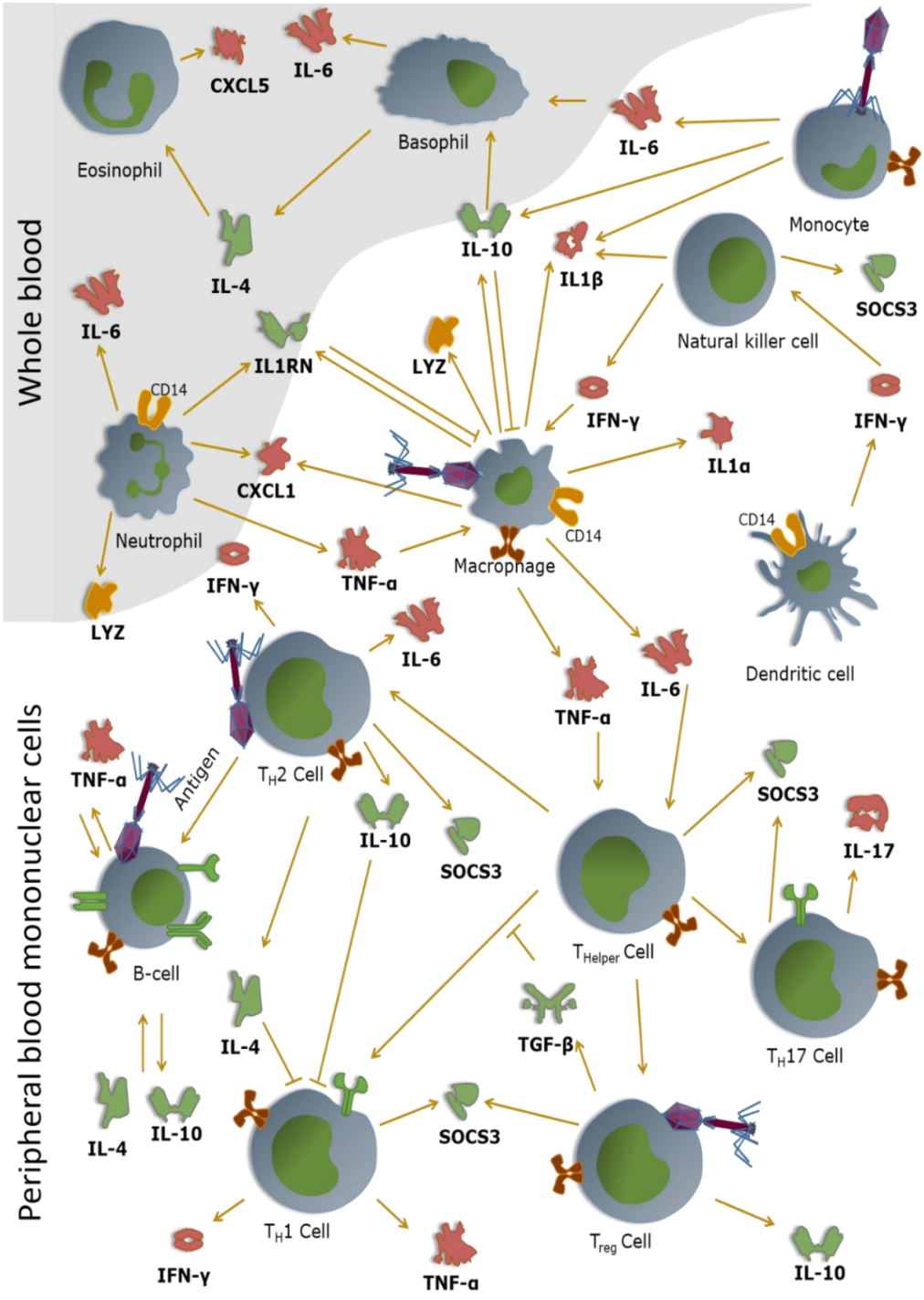
Culture of Pseudomonas aeruginosa and Staphylococcus aureus strains

Pseudomonas aeruginosa strain 573 (received from the Eliava IBMV, Tbilisi, Georgia) and *Staphylococcus aureus* strain ATCC 6538 were grown on a Lysogeny Broth (LB) agar plates (Becton Dickinson, Erembodegem, Belgium) and incubated overnight at 37 °C. One colony was subsequently used to inoculate a 15 ml tube containing a 4 ml LB agar slant, and incubated again overnight at 37 °C. Five ml saline was added to yield a suspension with a final concentration of 10^7 cfu/ml, as confirmed by culture of serial tenfold dilutions.

Bacteriophage preparation

Phage propagation: Bacteriophage stocks (Table 8. 1) were prepared using the double-agar overlay method as described in Merabishvili *et al.* (2009). Briefly, one ml of phage preparation containing 10^6 plaque forming units (pfu) of bacteriophages was mixed with 3 ml of molten (45 °C) LB top Bacto agar (0.6 %) (Becton Dickinson) and 100 µl of the host strain suspension (end concentration of 10^7 cfu/ml) in a sterile 14 ml tube (Falcon, Becton Dickinson). This mixture was plated onto freshly made 90 mm diameter Petri dishes (Plastiques Gosselin, Menen, Belgium), filled with a bottom layer (20 ml) of 1.5 % LB agar, and incubated aerobically at 32 °C for 16 h. Subsequently, 200 µl of chloroform was added to the lids of the Petri dishes and the inverted plates were further incubated at 4 °C for 1 h. The top layer of the double-agar layer was scraped off using a sterile Drigalski spatulum and transferred to a sterile 50 ml tube.

Figure 8. 6: Hypothetical representation of the interaction of bacteriophages with mammalian immune cells. Phages potentially interact with (currently unknown) immune receptors and induce corresponding immune responses. The immune responses induced by the bacteriophages can either be pro- of anti-inflammatory. For instance, the tested phages are able to induce the pro-inflammatory cytokines IL1α and IL1ß. Through the induction of IL1RN by the phage, the phage is able to inhibit the pro-inflammatory responses that are otherwise induced by these cytokines. Pro-inflammatory cytokines are marked in red (*i.e.* TNFα, IFN-γ, IL1α, IL1ß, IL6, IL17, CXCL1 and CXCL5), anti-inflammatory cytokines are depicted in green (*i.e.* TGFß, IL4, IL10 and IL1RN). Dark orange depicts proteins that play a key role in the removal or perception of bacterial pathogens (*i.e.* CD14 and LYZ).


Whole blood
Peripheral blood mononuclear cells
Eosinophil
CXCL5
IL-6
Basophil
IL-6
Monocyte
IL-10
IL1β
SOCS3
Natural killer cell
IL-4
IL-6
IL1RN
LYZ
CD14
IFN-γ
IFN-γ
IL1α
CXCL1
Neutrophil
CD14
Macrophage
IFN-γ
TNF-α
LYZ
CD14
Dendritic cell
IL-6
TNF-α
IL-6
TNF-α
Antigen
TH2 Cell
IL-10
SOCS3
SOCS3
IL-17
B-cell
THelper Cell
IL-4
TGF-β
TH17 Cell
IL-4
IL-10
SOCS3
TH1 Cell
Treg Cell
IFN-γ
TNF-α
IL-10

The harvested phages were centrifuged for 20 min at 6,000 x *g* at 4 °C. The supernatant was aspirated using a sterile 10 ml syringe (BD Plastipak, Becton Dickinson) with a 30 G sterile needle (BD Microlance 3, Becton Dickinson) and passed through a 0.22 µm membrane filter (Sartorius Stedim, Zellik, Belgium). The filtrate was subsequently centrifuged at 35,000 x *g* for one hour. The phage pellet was resuspended in 5 ml saline and stored at 4 °C overnight before determining the phage titer. Preferably, the titer of the phage lysate should be checked at least one day later according to the above described procedures. This will allow phage particles that may have clumped together during centrifugation steps to disengage (Kutter and Sulakvelidze, 2004).

Phage titer determination: The bacteriophage titer was determined by assaying decinormal serial dilutions (log(0) to log(-12)) of the bacteriophage suspension with the overlay method (Merabishvili et al., 2009). One ml of each dilution was mixed with 3 ml of molten (45 °C) LB 0.6 % top Bacto agar and the host strain (end concentration of 10^7 cfu/ml) in a sterile 14 ml tube. This mixture was plated in triplicate onto 90 mm diameter Petri dishes, filled with a bottom layer of 1.5 % LB agar, and incubated for 16 h at 37 °C. To determine the original bacteriophage concentration, plates with one to 100 distinguishable plaques were counted. The mean was then calculated for the triplicate plates.

Endotoxin removal: Endotoxins were removed and quantified as described in Van Belleghem *et al.* (2017). Briefly, phage lysates were further purified using CsCl centrifugation. Subsequently, endotoxin concentrations were determined using the EndoZyme recombinant Factor C (rFC) Assay (Hyglos, Bernried am Starnberger See, Germany). For the *P. aeruginosa* phages, 10^{-5} – 10^{-10} EU/pfu remained after the CsCl purification. The *S. aureus* phage ISP contained no endotoxins (Table 8. 1).

PBMC isolation

Peripheral Blood Mononuclear Cells (PBMCs) were isolated from a buffycoat after informed consent (Blood Transfusion Centre, Ghent), using a Lymphoprep (Axis-Shield, Dundee, Scotland) gradient. Fifty ml of the buffycoat was added to 250 ml Hank's Balanced Salt Solution, without Ca^{2+} and Mg^{2+} (HBSS) (Invitrogen). Of this dilution, eight aliquots of 35 ml were each added to 15 ml Lymphoprep in a 50 ml Falcon tube. These mixtures were subsequently centrifuged at 500 x *g* for 20 min at room temperature. The inner whitish ring of PBMCs, present between the lymphoprep and the plasma phase, was transferred to 25 ml HBSS and centrifuged at 450 x *g* for 10 min at room temperature. The supernatants was removed and the cell pellet was resuspended in 10 ml HBSS. All resuspended cells were pooled into a 50 ml Falcon tube and HBSS was added to a total volume of 50 ml. A small fraction of this cell solution was used to count the number of cells present, before it was centrifuged again at 350 x *g* for 10 min at room temperature.

The total number of cells was counted using a Sysmex KX-21 (Sysmex, Norderstedt, Germany). The cell pellet was resuspended in heat-inactivated fetal calf serum with

10 % dimethyl sulfoxide (DMSO) to a concentration of 2 x 10^7 cells/ml and divided in 1 ml aliquots before cryostorage them in liquid nitrogen.

Stimulation of PBMCs

Stimulations were performed in 100 µl volumes containing 10^6 PBMCs. One vial of stored PBMCs, containing 2 x 10^7 cells/ml, was thawed at 37 °C prior to adding 9 ml HBSS (without Ca^{2+} and Mg^{2+}). This suspension was subsequently centrifuged at 350 x *g* for 10 min. The obtained cell pellet was resuspended in 5 ml HBSS (without Ca^{2+} and Mg^{2+}) and 80 µl was used for cell counting on a Sysmex KX-21. This cell suspension was centrifuged at 350 x *g* for 10 min. The resulting cell pellet was resuspended in RPMI 1640 (supplemented with 2 mM L-glutamic acid, 1X MEM non-essential amino acids, 1 mM sodium pyruvate, 60 U of penicillin/ml, 10 mg/ml streptomycin, 2 mM L-glutamine and 10 % heat inactivated fetal calf serum) to a final concentration of 10^7 cells/ml (Moore et al., 1967).

The PBMCs (*i.e.* 10^6 PBMCs/100 µl) were subsequently stimulated with 10 µl of the bacterial host (*i.e.* either *P. aeruginosa* or *S. aureus,* at a concentration of 10^7 cfu/ml, *i.e.*, 10^5 cfu per 10^6 PBMC or 10^{-1} cfu/PBMC), 10 µl of the phage suspensions (*i.e.* either a phage lysate or a highly purified (CsCl) phage suspension (Table 8. 1)) at concentrations between 10^{13} and 10^9 pfu/ml, *i.e.*, 10^5 or 10 pfu/PBMC, or phage suspension in combination with 10 µl endotoxins (commercial preparation derived from *P. aeruginosa* strain 10; Sigma Aldrich) at concentrations of 10^1, 10^5 or 10^7 Endotoxin units/ml (EU/ml), *i.e.*, 10^{-1}, 10^{-3} or 10^{-7} EU/PBMC. As a negative control, 10 µl saline was added to the cells. The PBMCs were incubated for 20 h at 37 °C in 5 % CO_2. All stimulation experiments and controls were carried out on six biological replicates (*i.e.* anonymous donors) in triplicate. We confirm that all methods were carried out in accordance to relevant guidelines and regulations and that all experimental protocols were approved by the ethical committee of Ghent University (EC/2017/0558).

Extraction of total nucleic acids: After 20 h of PBMC stimulation, the total cell volume was transferred to 1 ml Qiazol (Qiagen, Valencia, CA) and stored at -80 °C for at least 16 h before extracting the RNA. The total RNA fraction was isolated using a semi-automated procedure of NucliSens EasyMag (Biomérieux, Marcy l'Étoile, France). Briefly, 900 µl EasyMag lysis buffer was added to the 1.1 ml Qiazol solution. This mixture was subsequently transferred to a NucliSens EasyMag cartridge. Finally, 100 µl magnetized silica was added and the cartridge was loaded on the machine (according to the manufacturer). In a final step, the nucleic acids was eluted with 35 µl NucliSens EasyMag elution buffer.

DNase digest: DNase digest was performed immediately after nucleic acid extraction to remove DNA from the sample. Five µl of the nucleic extract was added to one µl of DNase (1 U/µl), one µl 10X DNase reaction buffer (Promega, Mannheim, Germany) and three µl of RNase free H_2O to make up a total volume of 10 µl. This mixture was incubated for 30 min at 37 °C. The DNase digestion was terminated by

adding 1 µl of DNase Stop Solution and incubating the mixture at 65 °C for 10 min. DNase digested RNA samples were stored at -80 °C.

cDNA synthesis: The cDNA synthesis was preformed using the RevertAid RT kit (Thermo Scientific, Waltham, MA) in four times 40 µl volumes according to the manufacturer's instructions.

Gene expression analysis of 12 immunity-related genes

RT-qPCR: Based on the RNA seq data, twelve target genes were selected for further evaluation by RT-qPCR. Combining the datasets of both the phage PNM lysate and *P. aeruginosa* stimulated PBMCs; we observed that a total of 2679 genes were uniquely expressed. As a cut-off for qPCR detection, a minimum of 7000 reads was selected either in the control condition or after stimulation, to ensure detection in the RT-qPCR. This led to a reduction to 418 genes. Furthermore, to detect a difference in gene expression in the qPCR, a Log2Fold change difference was needed after normalization of the target genes by the reference gene *ACTB*. This led to a further reduction to 176 potential target genes for qPCR-based analysis. From these, a total of twelve genes were selected on the basis of their possible importance in the immune response, *i.e CD14, CXCL1, CXCL5, IL1A, IL1B, IL1RN, IL6, IL10, LYZ, SOCS3, TGFBI* and *TNFA* (Table 8. 2).

The RT-qPCR was performed in a total reaction volume of 10 µl using 0.5 µM of each primer (Table 8. **3**), 2.5 µl cDNA and LightCycler 480 High resolution Melting master (Roche Applied Sciences, Indianapolis, Indiana).

The selection of the most stable reference gene(s) was carried out as described by Vandesompele *et al*. (2002) using SybrGreen as means of detection. Using geNorm, we found that all six reference genes (*i.e. ACTB, GADPH, HRPT1, PPIA, TBP and UBC*; Table 8. 4) are sufficiently stable (internal control gene-stability measure M < 1.5). Therefore, the minimal optimal number of reference targets to be used in this experiment was 1 (V < 0.15). The mRNA levels are expressed in relative copy numbers normalized against the reference gene (ACTB), as described by Giulietti et al. (2001) and Samarasinghe et al. (2006).

Table 8. 2: Selection of the 12 target genes for further analysis by RT-qPCR.

Symbol	# reads control	# reads phage PNM lysate	# reads *P. aeruginosa* strain 573	Log2Ratio phage PNM lysate	Log2Ratio *P. aeruginosa* strain 573
TGFBI	34300	640	62	-5.68	-9.27
CD14	134382	NA	594	NA	-7.96
LYZ	42484	3997	206	-3.34	-7.83
CXCL5	249901	NA	8756	NA	-4.97
IL1B	30961	636519	137021	4.44	2.01
IL1RN	3167	41322	19681	3.78	2.5
TNF	2260	4823	16990	1.17	2.77
IL1A	418	28048	8740	6.15	4.25
IL10	363	6890	7771	4.32	4.28
IL6	573	51002	52356	6.55	6.37
SOCS3	20597	79658	NA	2.02	NA
CXCL1	20185	152399	NA	2.99	NA

Selection was based on number of reads in either control of stimulated condition (*i.e.* phage PNM lysate or *P. aeruginosa* strain PA573) which needs to exceed 6000 and the Log2ratio, which needs to be larger than |2|. NA indicated no differential expression for a specific condition/gene; hence no number reads were included.

Table 8. 3: List of primers used in the RT-qPCR for the validation of the RNA seq.

Names	Sequence (5' - 3')	Tm (°C)
IL10_F	CATCGATTTCTTCCCTGTGAA	45.3
IL10_R	TCTTGGAGCTTATTAAAGGCATTC	47.2
TNFa_F	CCCAGGGACCTCTCTCTAATC	51.2
TNFa_R	ATGGGCTACAGGCTTGTCACT	49.2
ACTB_F	GGATGCAGAAGGAGATCACTG	49.2
ACTB_R	CGATCCACACGGAGTACTTG	62.0
SOCS3_F	GGCCACTCTTCAGCATCTC	60.0
SOCS3_R	ATCGTACTGGTCCAGGAACTC	49.2
TGFBI_F	GAAGGGAGACAATCGCTTTAGC	49.7
TGFBI_R	TGTAGACTCCTTCCCGGTTGAG	51.6
CD14_F	CGCTCCGAGATGCATGTG	58.0
CD14_R	TTGGCTGGCAGTCCTTTAGG	62.0
LYZ_F	AAAACCCCAGGAGCAGTTAAT	45.3
LYZ_R	CAACCCTCTTTGCACAAGCT	60.0
CXCL5_F	ATCTGCAAGTGTTCGCCATAG	47.3
CXCL5_R	ACAAATTTCCTTCCCGTTCTTC	46.0
IL1B_F	GGCCACATTTGGTTCTAAGAAA	46.0
IL1B_R	TAAATAGGGAAGCGGTTGCTC	47.3
IL1RN_F	GAAGATGTGCCTGTCCTGTGT	49.2
IL1RN_R	CGCTCAGGTCAGTGATGTTAA	47.3
IL1A_F	CGCCAATGACTCAGAGGAAGA	49.2
IL1A_R	AGGGCGTCATTCAGGATGAA	60.0
IL6_F	GGTACATCCTCGACGGCATC	64.0
IL6_R	GCCTCTTTGCTGCTTTCACAC	49.2
CXCL1_F	GGAAAGAGAGACACAGCTGCA	49.2
CXCL1_R	AGAAGACTTCTCCTAAGCGATGC	50.2

The F denotes the forward primer and the R denotes the reverse primer.

Table 8. 4: Primers used to determine the most stable reference genes.

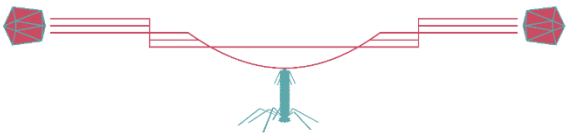
mRNA target	Name primer	Sequence (5'- 3')	Tm (°C)
ACTB	ACTB_F	GGATGCAGAAGGAGATCACTG	49.2
	ACTB_R	CGATCCACACGGAGTACTTG	62.0
HPRT1	HPRT1_F	TCAGGCAGTATAATCCAAAGATGGT	49.3
	HPRT1_R	AGTCTGGCTTATATCCAACACTTCG	50.9
UBC	UBC_F	TCGCAGCCGGGATTTG	52.0
	UBC_R	GCATTGTCAAGTGACGATCACA	47.9
TBP	TBP_F	ATGTGAAGTTTCCTATAAGGTTAG	45.4
	TBP_R	AGGAAATAACTCTGGCTCATAAC	46.6
GAPDH	GAPDH_F	TCACCACCATGGAGAAGGC	60.0
	GAPDH_R	GCTAAGCAGTTGGTGGTGCA	62.0

F denotes the forward primer and R denotes the reverse primer.

Chapter 9

In Silico Modeling:

The Phage - Bacteria - Immune response interaction



"Time is a drug. Too much of it kills you."

Terry Pratchett, Small Gods

Introduction

Phages as pharmaceuticals are protein-based, biological agents that actively replicate, and even evolve during manufacture or use and that can potentially interact with the body's immune system (Loc-Carrillo and Abedon, 2011). Phages may control bacterial infections in two ways. Under *active treatment* most of the bacteria are killed by secondary infections after extensive reproduction and transmission of the phage. If the phages do not increase in number, there can still be *passive treatment* in which the initial phage dosage is large enough to inundate the bacteria by primary infection alone or by lysis from without, whereby the bacterial cell loses its integrity due to membrane damage caused by massive phage adherence and penetration. Kinetically, passive treatment can be compared to antibiotic treatment, both lacking the advantage of amplification of the antibacterial agent, as is the case in active treatment, when secondary phage infections do occur (Payne and Jansen, 2000).

When pharmaceutical agents are active replicating entities, a different set of pharmacokinetic principles is needed. Furthermore, these entities can interact, replicate and evolve, and demonstrate phenomena unknown in chemical kinetics of conventional drugs (Payne and Jansen, 2003). The specific pharmacokinetic phenomena expected to occur when actively replicating viruses are used to control bacterial infections have been described (Cairns et al., 2009; Kasman et al., 2002; Payne and Jansen, 2001, 2003, 2000). In these mathematical models, the rate at which a bacterial population declines due to phage infection, the rate at which the phage population increases and the levels at which they are maintained depends primarily on five parameters: the infectivity of the phage, the latency period, the burst size, the rate at which the phages are degraded or removed from the site of infection, and the bacterial growth rate. Besides these five parameters, two other variables need to be taken into account: the density of susceptible bacteria and the density of phage (Levin and Bull, 2004). In summary, these models describe phage pharmacodynamics as being analogous to the population dynamics of the phage-bacterial interaction (Abedon and Thomas-Abedon, 2010), not taking into account potential interaction between bacteria and/or phages with the innate or adaptive immune response.

Hodyra-Stefaniak *in silico* model

Recently, these mathematical models have been further extended to include the mammalian host response towards the phage (Hodyra-Stefaniak et al., 2015). Based on experimental data, the authors developed a general scheme of the tripartite interactions between bacteriophages, bacteria and mammalian immunity. This scheme summarizes the main reciprocal dependencies, specifically the effect that limit the bacterial or phage survival, or the effects inducing the activation of the immune response (Figure 9. 1). There are three key assumptions on which their scheme is based. First, adaptive immunity specific to phages and adaptive immunity specific to bacteria have no important cross-talk; second, phages are not

able to boost an innate immune response (Miernikiewicz et al., 2013; Park et al., 2014); and third, the boosted innate immune response acts against the bacteria, but at the same time also acts against the phage. These assumptions led to the development of a mathematical model based on those previously described by Levin and Bull (Levin and Bull, 1996, 2004), and Payne and Jansen (Payne and Jansen, 2001, 2003). This led to the development of a model with a set of immunology-representing variables; innate immunity (I), adaptive immunity specific to phages (A), and adaptive immunity specific to bacteria (B) (Addendum – Mathematical model).

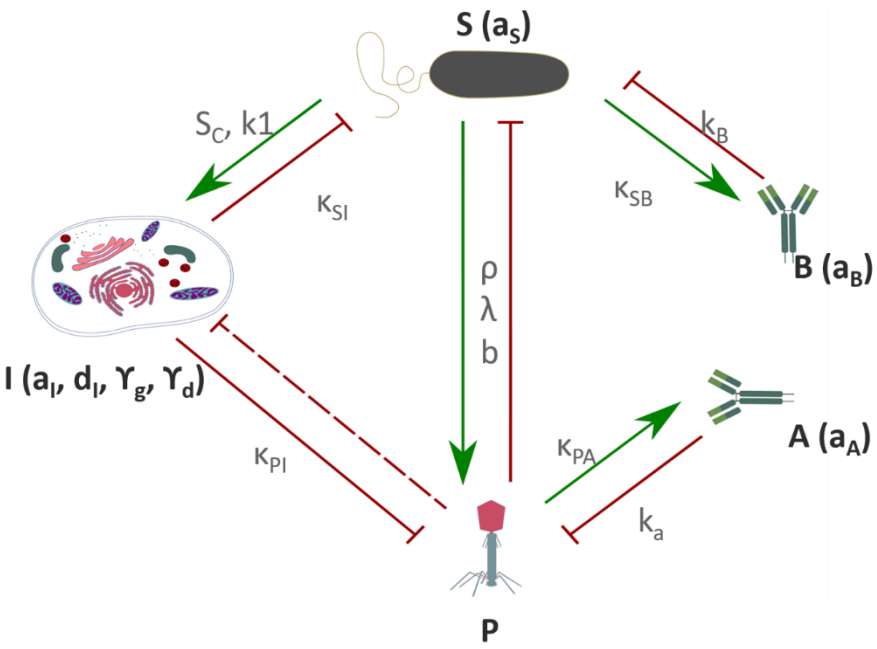


Figure 9. 1: Schematic representation of the immune response against phages and bacteria. P – Phage, S – susceptible bacteria, I – innate immunity, A – adaptive immune response to phage, B – adaptive immune response to bacteria. Green arrows represent a stimulatory effect; red arrows represent an inhibitory effect. Adapted from Hodyra-Stefaniak et al. (2015) with the addition of the anti-inflammatory property of the phage (dotted line). Abbreviations are further explained in addendum Table A 1.

The Hodyra-Stefaniak *in silico* model predicts a successful intervention of phages in the removal of a bacterial infection when no interaction occurs between the innate immunity and the phages (Figure 9. 2A). Similar outcomes were predicted in an *in silico* model developed by Leung and Weitz (2017). This *in silico* model also does not include a direct interaction of phages with the innate immune response, but hints

to the importance of neutrophils in order to remove the remainders of the bacterial infection or the occurrence of phage resistant bacteria. The inclusion of the innate immunity-representing variable in the Hodyra-Stefaniak *in silico* model (Addendum – Mathematical model) demonstrates that the expected outcome of phage therapy could be abrogated by the innate immunity boosted by the bacteria (Figure 9. 2B). Moreover, the removal of the phage leads to a second increase in bacterial count. Subsequently, the phage therapeutic intervention fails, in contrast to phage therapy related data available (Chapter 5). According to this model, the undesirable effect could be counteracted by adjusting the phage dose or altering the timing, as long as the innate immunity is considered (Figure 9. 2D). These *in silico* models could eventually be further optimized, to more closely mimic the immunological outcome, by including the innate immune response induced by the phage. Whether the phage-induced immune response is dependent on the bacterial presence needs to be further evaluated, as such data are limited.

Updating the Hodyra-Stefaniak *in silico* model

The Hodyra-Stefaniak and Leung *in silico* models miss one key feature: the interaction of the phage with the innate immune response. This interaction can be anti-inflammatory, *i.e.* a suppression of the immune response, or pro-inflammatory, *i.e.* an increase of the immune response.

Our results indicate that phages are able to induce innate immune responses and more specifically that they have anti-inflammatory properties (Chapter 8). Therefore, the *in silico* model of Hodyra-Stefaniak *et al.* (2015) could be further adapted to include an innate component effected by the phages. If the phage has anti-inflammatory properties, the innate immune response should decrease. This should provide the phage with a higher survival rate. Therefore, we suggest to expand the equation of the innate immune response (Addendum – mathematical model - Equation 3), to include the effect of phages on the innate immune response (X_P). We propose to divide the initial equation describing the innate immune response (I) into two parts (Addendum – Equation 4-9), one part describing the effect of bacteria on the innate immune response (X_S; Addendum – Equation 4-6) and the other one describing the effect of phages on the innate immune response (X_P; Addendum – Equation 7-9). Furthermore, additional constraints were given to the mathematical model. When the phage concentration (P) is larger than the critical phage concentration (P_C), *i.e.* the phage concentration needed to induce an anti-inflammatory response and to reduce the innate immune response, the phage decay rate (Υ_{dP}) approaches one.

Although only limited data are available about the interaction of phages with the innate immune response, the modified Hodyra-Stefaniak model more closely resembles a real-life outcome, but still forms a hypothetical model. Our adapted model thus describes the effect of innate and adaptive immunity on the success or

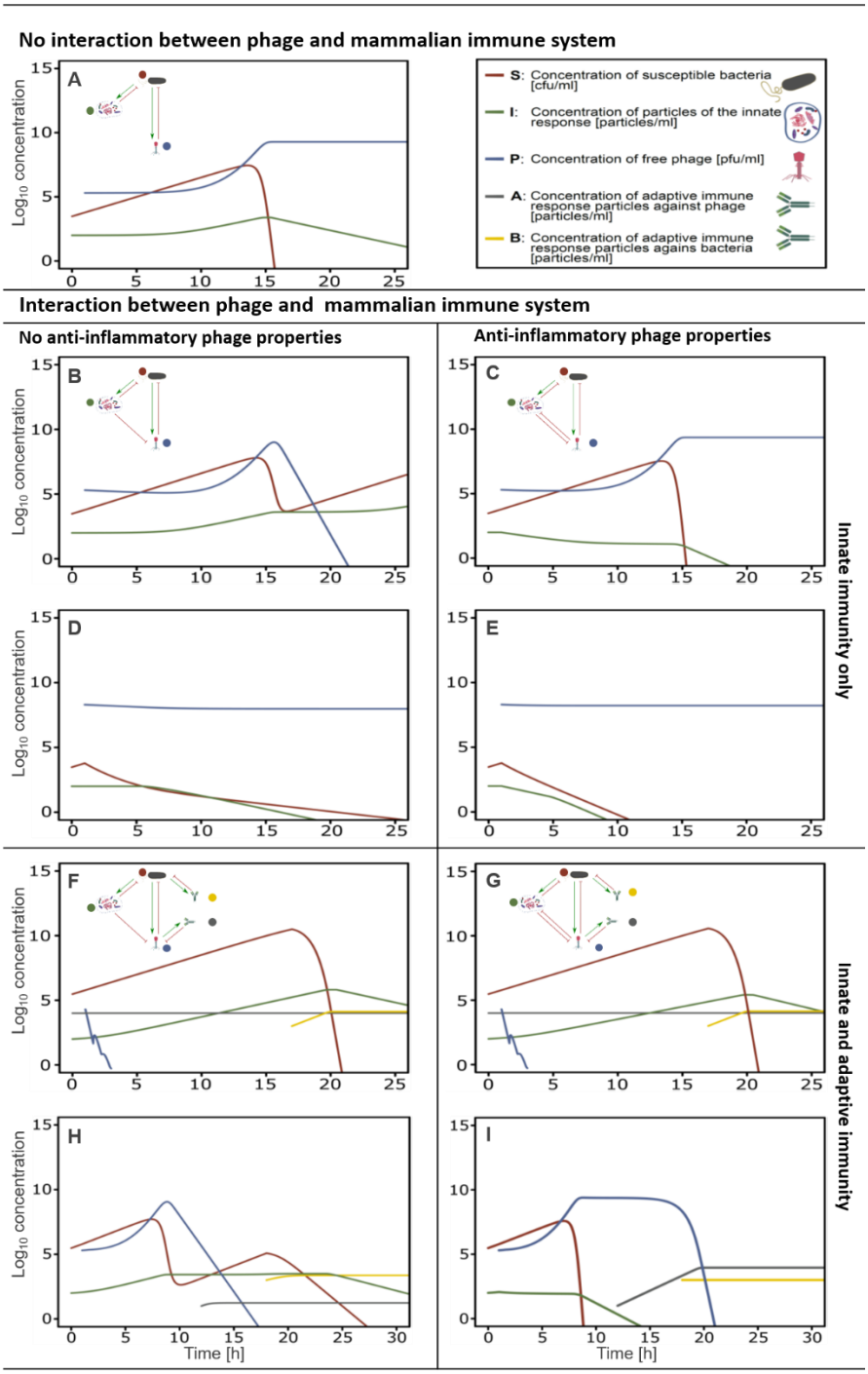
failure of phage antibacterial treatment, taking into account the anti-inflammatory nature of the phage (Figure 9. 2C, E, G and I). By including the anti-inflammatory property of phages to the innate immunity equation the prediction of the phage therapeutic outcome becomes successful again (Figure 9. 2C), in contrary to what the initial model predicts (Figure 9. 2B). The phage is able to subdue the innate immune response, subverting its rapid removal and hence clears the bacterial infection. When a bacterial infection is combated with an initial high phage dose, the effects of the innate immune response are negligible (Figure 9. 2D and E). Yet, if the phage has anti-inflammatory properties, the bacterial clearance occurs much faster. Nevertheless, if anti-phage antibodies are present prior to the phage therapeutic intervention, the intervention fails as the phages are rapidly removed (Figure 9. 2F and G).

When no pre-immunization is present, and no anti-inflammatory phage properties are taken into account, the removal of the bacterial infection is attributed to the adaptive immune response against the bacteria (Figure 9. 2H). Initially the phages lead to a reduction of the bacterial count, but are themselves removed by a combination of the innate and adaptive immune response towards the phage. This leads to a second rise in the bacterial concentration. In a later stage the bacterial infection is removed by the adaptive immune response directed against the bacteria, hence the clearance of the bacterial infection is not due to the presence of the phage but due to the adaptive immune response against the bacteria. When assuming anti-inflammatory properties of the phages, the bacterial infection is cleared much faster and is attributed to the presence of the phage (Figure 9. 2I).

Conclusion

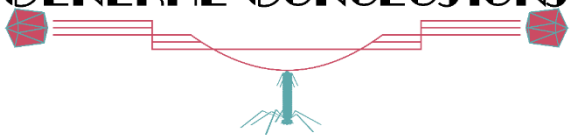
The expanded hypothetical model clearly shows that, although the adaptive immunity is an important factor in the success or failure of phage antibacterial therapy, the anti-inflammatory properties of the phage could explain why the treatment of a bacterial infection is successful (Figure 9. 2C and I). Additionally, our model predicts that an anti-inflammatory effect of phage is necessary (Figure 9. 2C and I), otherwise phage therapy could not be successful (Figure 9. 2B and H). We can conclude that phages have an effect on the human immune response, but this is often overshadowed by the negative effects induced by the bacterial infection.

Figure 9. 2: Effects of innate and adaptive immunity on the success or failure of phage antibacterial treatment, numerical simulations. Innate immune response. A| No relation between innate immunity and phage viability. The survival of the phage is independent of the presence of an innate immune response. **B| Phage susceptibility to the innate immune response.** The innate immunity has a negative effect on the phage survival and leads to its removal. Subsequently the bacteria are no longer infected by the phage, and a rise in bacteria is observed. **C| Phage susceptibility to the innate immune response, taking into account the anti-inflammatory property of the phage.** The anti-inflammatory characteristic of the phage leads to a decline in innate immune particles. This has as effect that the bacterial count diminish and the phage survives, similar to A. **D| Phage susceptibility to innate immune response accommodated and counteracted by an increased phage dose.** The higher phage dose leads to the removal of the pathogen and the survival of the phage. **E| Phage susceptibility to innate immune response accommodated and counteracted by an increased phage dose, assuming the anti-inflammatory property of the phage.** The effect is the same as in D, but the innate immune response is diminished. Innate and adaptive immune response. **F| Phage susceptibility to the innate immune response and presence of pre-immunization towards the phage.** Presence of pre-existing anti-phage antibodies leads to a rapid drop in phage concentration, hence the phages have no effect on the survival of the bacteria. Once an adaptive immune response against the bacteria is present, the bacterial count decreases. **G| Phage susceptibility to the innate immune response and no pre-immunization to the phage exists, assuming the anti-inflammatory property of the phage.** The anti-inflammatory response of the phage has no direct influence on the phage survival in the presence of an adaptive immune response against the phage. Overall, the response is similar as in F. **H| Phage susceptibility to the innate immune response and no pre-immunization to the phage exists.** The absence of a specific adaptive immune response against the phage leads to a decrease in the bacterial population. The combined effect of innate and adaptive immunity towards the phage leads to a drop in phage particle concentration. **I| Phage susceptibility to the innate immune response and no pre-immunization to the phage exists, assuming the anti-inflammatory property of the phage.** Once the phage reaches a critical concentration (Pc, the concentration of phages needed to induce an anti-inflammatory response), the innate immune response decreases and the phage concentration grows until all bacteria are removed. Once an adaptive immune response is present against the phage, the phage concentration diminishes until completely removed. Variables and parameters used in these models are described in Addendum – Mathematical model – Table A1. The Y-axis represents the respective particle concentration and the X-axis represents the time, in hours.


No interaction between phage and mammalian immune system
A
Log10 concentration
S: Concentration of susceptible bacteria [cfu/ml]
I: Concentration of particles of the innate response [particles/ml]
P: Concentration of free phage [pfu/ml]
A: Concentration of adaptive immune response particles against phage [particles/ml]
B: Concentration of adaptive immune response particles agains bacteria [particles/ml]
Interaction between phage and mammalian immune system
No anti-inflammatory phage properties
Anti-inflammatory phage properties
B
C
D
E
F
G
H
I
Innate immunity only
Innate and adaptive immunity
Time [h]

Chapter 10

General Conclusions



"An expert is a person who has made all the mistakes that can be made in a very narrow field."

Niels Bohr

With the growing interest in phage therapy, due to the rising prevalence of antibiotic resistant bacteria, clinical trials are being set up to evaluate the efficacy and safety of this *new* type of therapeutic intervention (Levy and Marshall, 2004; Matsuzaki et al., 2014; Merabishvili et al., 2009; Rhoads et al., 2009; Wright et al., 2009). Most of these available phage therapy trials primarily address safety issues of topically or orally applied phages.

Phages, as pharmaceuticals, possess unique pharmacokinetics and pharmacodynamics that are generally poorly understood (Cooper et al., 2016). To further understand these pharmacokinetic and –dynamic properties it is important to perform *in vivo* and *in vitro* experiments. Furthermore, to perform such studies, it is of vital importance that the phage preparations are free of any contaminating molecules such as bacterial proteins or endotoxins, released during phage production.

Unpublished data from the Barr lab indicates that phages possess the ability to transcytose from the apical-to-basal side of different cell types (*i.e.* cell lines originating from the gut, lung, liver, kidney and brain). This clearly demonstrates that phage are able to cross cell layers and provides further evidence into how phages are able to interact with the mammalian immune system.

Endotoxin removal

Endotoxin removal from phage preparations is one of the most important, but also highly cumbersome, steps in the use of phages in therapeutic settings. Furthermore, the removal of endotoxins is important when studying the effects of phages on eukaryotic cells, more specifically; peripheral blood mononuclear cells (PBMCs). Recently, several methods have become available for the purification of phage preparations, taking into account the removal of endotoxins, such as *phage on tap* (Bonilla et al., 2016). This protocol described a quick and efficient method for the preparation of homogenous phage stocks. Unfortunately, this method requires specific lab equipment, *e.g.* speed vacuum to remove residual organic solvent. Our results showed a low endotoxin removal efficacy when organic solvent (1-octanol) was used, this might be due to differences in the phages used (whereas we used *P. aeruginosa* phages, Bonilla used *E. coli* phages).

We evaluated the efficiency of seven different endotoxin purification strategies, (*i.e.*, Endotrap HD column purification and/or CsCl density centrifugation in combination with Endotrap purification, followed by organic solvent treatment, detergent treatment, enzymatic inactivation of the endotoxin using alkaline phosphatase, or CIM monolithic anion exchange chromatography) for five different phages (Chapter 6 – Endotoxin purification strategies). Interestingly, large differences with regard to initial endotoxin concentrations were observed for the four different *P. aeruginosa* phages, ranging from approximately 10^4 to 10^6 EU/ml (Van Belleghem et al., 2017a). The removal of endotoxins by CsCl centrifugation leads to an endotoxin removal efficacy of up to 99.6 %. Further removal of the remaining

endotoxins by additional strategies, such as the use of Endotrap HD, organic solvent, Triton-X100, alkaline phosphatase or anion-exchange chromatography enables a further reduction of the endotoxin content and a total endotoxin removal efficacy of 99.9 %.

Although we were not able to include the endotoxin content of the original phage lysates, our results do show the difficulties related to obtaining endotoxin-free *P. aeruginosa* phage preparations. Even though phages infecting the same bacterial host seem similar, they clearly showed different endotoxin removal efficacies. This makes it hard to provide a universal strategy for the removal of endotoxins from phage preparations, although a combination of strategies might be advised for the complete removal of endotoxins.

Comparing the endotoxin removal efficacy observed in our experiments with those described in the literature (which are often less than 10^2 EU/ml), the difference could be explained by the use of different phages. We mainly used *P. aeruginosa* phages whereas others have focused on *E. coli* phages (Bonilla et al., 2016; Boratynski et al., 2004; Szermer-Olearnik and Boratyński, 2015), or used repeated purification to obtain low endotoxin concentrations (Dufour et al., 2016).

For therapeutic purposes, phage titers of $10^7 – 10^8$ pfu/ml are used (Merabishvili et al., 2009). When starting from phage preparations of 10^{12} pfu/ml, the low endotoxin removal efficacy can be supplemented with further diluting the phage preparation. In our case this would lead to a further reduction in the endotoxin concentration ranging from 0.0002 to 31.6 EU/ml for 10^7 pfu/ml, which is within the therapeutically allowed range of 5 EU/ml/kg/h for therapeutic applications (Daneshian et al., 2006). For example, a patient weighing 75 kg can be treated with over a six hour period with a 10^7 pfu/ml phage preparation containing a maximum amount of 30 EU/ml.

For other purposes, such as studying the immune responses induced by phages, lower endotoxin concentrations are advised without lowering the phage concentration. In case this is not possible, including control conditions with the same endotoxin concentration can be included, as was done in Chapter 8. Although this might complicate the analysis of the observed immune response, it enables one to establish to what extent the immune response is influenced by the residual endotoxins.

Immunomodulation by bacteriophages

It has been demonstrated that the oral uptake of phages by animals results in the translocation of phages to systemic tissues (Duerr et al., 2004; Hamzeh-Mivehroud et al., 2008; Majewska et al., 2015). This suggests that mammals have mechanisms for the uptake and delivery of phages. The resulting contact between systemic tissues and phages may allow intestinal phages to elicit innate and adaptive immune responses.

Therefor we evaluated the innate immune response induced by five different phages, *i.e.* four infecting *P. aeruginosa* and one infecting *S. aureus.* These five phages represent the three major phage morphologies: *Myoviridae, Podoviridae* and *Siphoviridae*. The initial immune responses were observed after 20 h of PBMC stimulation. To obtain a more in-dept view of the phage induced immune response, time course experiments could be conducted.

Based on the fact that phages are present everywhere, and translocation of phages from the gut to the blood has been shown (Duerr et al., 2004; Hamzeh-Mivehroud et al., 2008; Majewska et al., 2015), it is not surprising that they elicit immune responses (Figure 8. 6). Moreover, the four *P. aeruginosa* phages tested have an overall comparable induced immune response, which largely corresponds to our previously performed transcriptome analysis of PBMCs stimulated with a *P. aeruginosa* phage PNM lysate (Van Belleghem et al., 2017b). Furthermore, the immune response induced by the Gram-negative *P. aeruginosa* phages is similar to the one induced by the Gram-positive *S. aureus* phage ISP. Although small differences in the expression of *CD14, IL10, TGFBI* and *LYZ* were observed (Figure 8. 1 and 8. 4), this could be attributed to the difference in host bacteria instead of difference in the phage morphology, as the four *P. aeruginosa* phages belong to different phage morphologies.

The removal of all endotoxins from the *P. aeruginosa* phages was not possible, leaving up to 10^6 EU/ml present in the phage preparations in the phage lysates. Therefore, we compared the immune response induced by the phage lysate and the highly purified phage, with the addition of endotoxins in the same concentration as present in the phage lysate. We observed that the immune response of the highly purified phage with addition of endotoxins did not bring it more closely to that of the phage lysate (Figure 8. 3). Furthermore, the addition of endotoxins to a highly purified *S. aureus* phage ISP preparation did not alter the observed immune response previously induced by the phage lysate or the highly purified phage preparation.

This indicates that the observed immune response is endotoxin independent for the twelve immunity related genes evaluated during our studies. Our results indicate that phages can induce an anti-inflammatory response, as clearly shown by the up-regulation of *SOCS3* and *IL1RN*, which is a known inhibitor of the pro-inflammatory IL1 response (Arend, 2001).

Although our study of the phage induced immune response is based on direct gene expression and not protein production, our data clearly show that phages are able to induce an immune response. In order to further validate these observed response, cytokine protein productions could be measured overtime.

In silico model of phage – bacteria – immune response interaction

The Hodyra-Stefaniak *in silico* model, not taking into account the possibility that phages might have anti-inflammatory properties, comes to the unexpected conclusion that phages would be inactivated by components of either the innate or adaptive immune response. This suggest that phages would not contribute to the clearing of the bacterial infection. This seems to contradict the well-established successes of many therapeutic applications of phages.

Expanding the Hodyra-Stefaniak *in silico* model (Hodyra-Stefaniak et al., 2015), with the inclusion of the anti-inflammatory property of phages, predicted a successful phage therapeutic intervention. Based on the results obtained during this PhD thesis, and the mathematical predictions of this model, indicating that a reduction on the innate immune response is necessary for the phages to remove a bacterial infection, we conclude that phages can interact with the mammalian innate immune response and that these interactions have important effects on the outcome of a phage therapeutic intervention. These *in silico* models can be further extended to include the occurrence of phage resistant bacterial strains during the phage therapeutic intervention, which will significantly affect the predicted outcome (Leung and Weitz, 2017).

Future perspectives

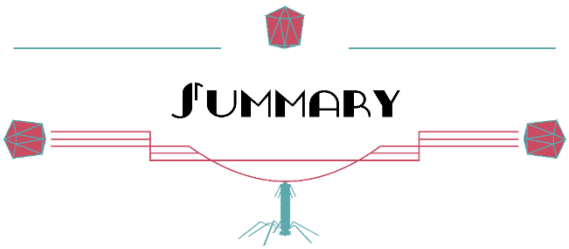
Based on the *in silico* model (Chapter 9); our experiments can be further extended to include the bacterial host. If it can be further consolidated that phages are able to induce anti-inflammatory responses, as our data suggests, this should lead to a successful intervention and removal of the bacterial infection. Moreover, could the phage induced anti-inflammatory properties influence the survival of the bacteria, when phage resistant strains are used? Are phages able to influence the immune response in such a way that it affects the bacterial survival? To what extent might the anti-inflammatory effect of phages overcome the pro-inflammatory response induced by the bacteria? Is this anti-inflammatory response active during the whole phage infection or only at certain time points or phage concentration? Could the anti-inflammatory properties of the phages dampen the unwanted side effects of an overreacting immune response? And could they in this sense reduce tissue damage in chronic infections, such as in the case of cystic fibrosis lung infection?

Answers to these questions should be able to give us a more in-depth view of what is occurring during phage therapy and might give us a better understanding why certain phage therapeutic interventions are more successful than others. Furthermore, separation of the immunological cells after stimulation with the phages could provide detailed information into which cells interact with the phages and induced the pro- and/or anti-inflammatory properties.

Whether phages induce a highly comparable overall immune response or whether only specific parts of the immune response are similar could be further studied through whole transcriptome analysis. Furthermore, time dependent studies

should be able to give us a more detailed view of the phage-induced immune response. Which time points are predominantly pro-inflammatory and which are predominantly anti-inflammatory. Moreover, which phage proteins are responsible for these anti-inflammatory responses? By comparing the immune response of a large set of phages, it will become possible, based on the phage genomes, to deduce the protein(s) responsible for these immune responses. Once these proteins are identified, their properties can be exploited to produce new anti-inflammatory drugs or to engineer new phages with elevated anti-inflammatory responses to increase the success rate of phage therapy.

It is thus important to gather more data concerning the pharmacodynamics and -kinetics of phages in an immunological context and further update these *in silico* models so they can be used to predict the outcome of a phage therapeutic intervention, such as determining the exact minimal concentration of phages needed in order for them to induce anti-inflammatory responses.



SUMMARY

Bacteriophages are the most abundant biological entities on Earth with an estimated number of up to 10^{30} particles. Every bacterial cell can potentially harbor many phages. These viruses use bacteria as a mean to replicate, almost always destroying their prokaryotic host in the process. Current knowledge states that phages ignore every cell but the strain of bacteria they have evolved to inhabit. This makes them ideal candidates to treat bacterial infections, while being harmless to mammalian cells and even non-target bacteria.

Phages provide a valid alternative to antibiotics. Nevertheless, the effect of phages on the human immune response needs to be evaluated. Therefore we need to obtain highly purified phage preparations, free of endotoxins. The removal of these endotoxins is necessary as they are highly immunogenic (as highlighted in Chapter 4) and could influence the induced immune response. For this purpose, we evaluated the endotoxin removal efficacy of seven different endotoxin removal strategies, described in Chapter 6. These strategies consisted of Endotrap HD column purification and/or CsCl density centrifugation in combination with Endotrap purification, followed by organic solvent (1-octanol) treatment, detergent (Triton X-100) treatment, enzymatic inactivation of the endotoxin using alkaline phosphatase, or removal of the endotoxin using CIM monolithic anion exchange chromatography, carried out for five different phages (*i.e.* four *P. aeruginosa* phages and one *S. aureus* phage).

We showed that CsCl density purification of the *P. aeruginosa* phages, at an initial concentration of 10^{12} - 10^{13} pfu/ml, led to the strongest reduction of endotoxins, with an endotoxin removal efficacy of up to 99.6 %, whereas additional purification methods yielded an additional endotoxin removal efficacy of 23 to 99 % on top of the initial purification, although sometimes accompanied with strong losses in phage titer.

Phage biology studies necessitate highly purified phage particles, when used in high concentration. Using the highly purified (CsCl density centrifugated, followed by Endotrap purification), endotoxin free, phage preparations, described in Chapter 6, we were able to study the immune response induced by these phages.

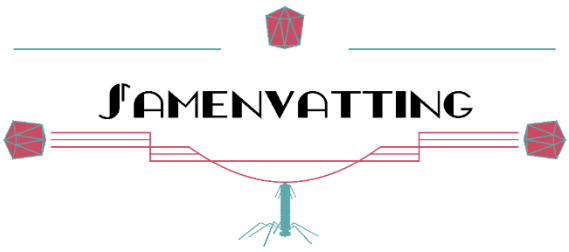
Chapter 7 describes a transcriptome analysis of freshly isolated peripheral blood mononuclear cells (PBMCs), isolated from one healthy individual, stimulated for 20 h with either a *P. aeruginosa* phage PNM lysate or its bacterial host *P. aeruginosa* strain 573. The phage PNM lysate was shown to increase the production of *IL10, IL6, SOCS1, SOCS3, CXCL2, CXCL3* and *CXCL6* and decrease the production of *LYZ, HLA-DMA, HLA-DMB, HLA-DRB1 and HLA-DRB4, CCL17, CCR1, CCR2* and *CCR5*. These results showed that the *P. aeruginosa* phage PNM lysate possess the potential to induce an immune response.

Further analysis on the potential of phages to induce an immune response was obtained by evaluating five different phages (*S. aureus* phage ISP and four *P.*

aeruginosa phages (*i.e.* PNM, LUZ19, 14-1 and GE-vB_Pae-Kakheti25)), discussed in Chapter 8. By means of specific RT-qPCR assays, we assessed the gene expression profile of PBMCs derived from six donors for twelve immunity-related genes (*i.e. CD14, CXCL1, CXCL5, IL1A, IL1B, IL1RN, IL6, IL10, LYZ, SOCS3, TGFBI* and *TNFA*). It was established that the phages were able to induce clear and reproducible immune responses.

First, the five different phages induced a comparable immune response, although endotoxins could not be completely removed from the *P. aeruginosa* phage preparations. Second, the observed immune response was largely anti-inflammatory, indicated by at least a fivefold up-regulation of IL1RN, IL10 and SOCS3. Third, the observed immune response was shown to be endotoxin-independent. Addition of endotoxins to the highly purified phages did not cause an immune response comparable to the one induced by the (endotoxin containing) phage lysate, but remained similar to that of the initial highly purified phage.

Using the observations made during these studies, we were able to construct a predictive hypothetical model on the outcome of phage therapy based on a previous model described by Hodyra-Stefaniak *et al.* (2015), described in Chapter 9. We expanded their initial model with the inclusion of an anti-inflammatory phage. This adaptation predicts important consequences on the theoretical outcome of a phage therapeutic intervention. Our model showed that when the phage has anti-inflammatory properties, phage therapy succeeds whereas the initial model showed a failure of the phage therapeutic intervention into the removal of the bacterial infection.



Samenvatting

Bacteriofagen, bacteriële virussen, zijn de meest voorkomende biologische entiteiten op aarde, met een geschat aantal van 10^{30} partikels. Elke bacteriële cel heeft de mogelijkheid om fagen voort te brengen. Deze virussen gebruiken de bacteriën om zich te repliceren. Dit proces heeft als gevolg dat de prokaryote gastheer bijna altijd wordt vernietigd. Gebaseerd op de literatuur wordt er in het algemeen gezegd dat fagen elke cel negeert buiten de bacteriële stam waarvoor ze geëvolueerd zijn om te kunnen infecteren. Dit maakt van hen ideale kandidaten om bacteriële infecties te behandelen, terwijl ze onschadelijk zijn voor niet-target bacteriën en zelfs eukaryote cellen.

Fagen vormen een belangrijk alternatief voor antibiotica. Desalniettemin is het belangrijk of de interactie van fagen met het humaan immuun systeem te evalueren. Daarvoor dienen we zeer zuivere, endotoxine vrije, faagbereidingen te bekomen. Het verwijderen van deze endotoxines is noodzakelijk omdat ze sterk immunogeen zijn (zoals aangehaald in Hoofdstuk 4) en zouden de geïnduceerde immuun respons kunnen beïnvloeden. Voor deze reden hebben we de endotoxine verwijderingseffectiviteit van zeven verschillende endotoxine verwijdering strategieën geëvalueerd, beschreven in Hoofdstuk 6. Deze strategieën omvatten een Endotrap HD kolom zuivering en/of CsCl densiteit centrifugatie in combinatie met Endotrap zuivering, gevolgd door een behandeling met organisch solvent (1-octanol), detergent (Triton X-100) behandeling, enzymatisch inactiveren van het endotoxine door gebruik te maken van alkalisch fosfatase, of een verwijdering van endotoxines door gebruik te maken van CIM monolithische anion uitwisseling chromatografie, uitgevoerd voor vijf verschillende fagen (*i.e.* vier *P. aeruginosa* fagen en één *S. aureus* faag).

We hebben kunnen aantonen dat CsCl densiteit centrifugatie van *P. aeruginosa* fagen, met een initiële concentratie van 10^{12} – 10^{13} pfu/ml, leidde tot de sterkste reductie in endotoxine concentratie, met een endotoxine verwijderingseffectiviteit van 99.6 %. Additionele zuiveringsmethodes leidde niet tot de volledige verwijdering van endotoxines uit de faag bereidingen, en leidde slechts tot een extra endotoxine verwijderingseffectiviteit van 23 tot 99 %, dit ging soms gepaard met een sterk verlies in faag concentratie.

Faag biologische studies hebben nood aan hoog zuivere faag partikels in hoge concentraties. Gebruikmakend van de hoog zuivere (CsCl ultra gecentrifugeerde, Endotrap gezuiverde), endotoxine vrije, faag bereidingen, beschreven in Hoofdstuk 6, waren we instaat om de immuun respons te bestuderen geïnduceerd door deze fagen.

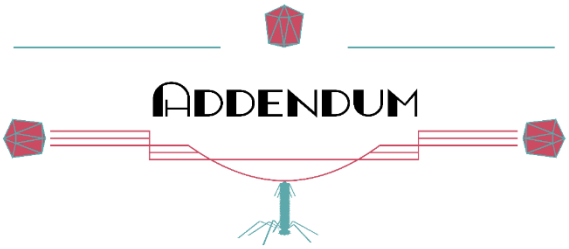
Hoofdstuk 7 beschrijft een transcriptoom analyse van vers geïsoleerde perifere bloed mononucleaire cellen (PBMCs), geïsoleerd uit één gezonde individu, gestimuleerd voor 20 h met ofwel een *P. aeruginosa* faag PNM lysaat of zijn bacteriële gastheer *P. aeruginosa* stam 573. De faag PNM lysaat verhoogde de genexpressie van de

genen *IL10, IL6, SOCS1, SOCS3, CXCL2, CXCL3* en *CXCL6* en verlaagde de genexpressie van *LYZ, HLA-DMA, HLA-DMB, HLA-DRB1, HLA-DRB4, CCL17, CCR1, CCR2* en *CCR5*. Deze resultaten toonde aan dat de *P. aeruginosa* faag PNM lysaat het potentieel bezit om een immuun respons te induceren.

Verdere analyse in verband met de mogelijkheid van fagen om een immuun respons te induceren werd verkregen door vijf verschillende fagen (een *S. aureus* faag ISP en vier *P. aeruginosa* fagen (*i.e.* PNM, LUZ19, GE-vB_Pae-Kakheti25 en 14-1)) te evalueren, besproken in **Hoofdstuk 8**. Door middel van een specifieke RT-qPCR assay bestudeerden we het genexpressie profiel van perifere bloed mononucleaire cellen, geïsoleerd uit zes verschillende donoren, voor twaalf immuniteit gerelateerde genen (*i.e. CD14, CXCL1, CXCL5, IL1A, IL1B, IL1RN, IL6, IL10, LYZ, SOCS3, TGFBI* en *TNFA*). Het werd aangetoond dat fagen in staat zijn om een duidelijke en reproduceerbare immuun respons te induceren.

Eerst en vooral, de vijf verschillende fagen induceren een vergelijkbare immuun respons, alhoewel alle endotoxines niet volledig verwijderd konden worden uit de *P. aeruginosa* faag bereidingen. Ten tweede, de geobserveerde immuun respons was hoofdzakelijk anti-inflammatoir, dit werd aangetoond door een vijfvoudige opregulatie van IL1RN, IL10 en SOCS3. Ten derde, er werd aangetoond dat de geobserveerde immuun respons endotoxine onafhankelijk was. Het toevoegen van endotoxines aan de sterk gezuiverde fagen zorgde er niet voor dat de geobserveerde immuun respons sterker lijkt op die van het (endotoxine bevattende) lysaat, maar bleef gelijkaardig aan dat van het initieel sterk gezuiverde faag preparaat.

Gebruikmakende van de observaties gemaakt gedurende deze studies waren we in staat een hypothetisch model te generen die de uitkomst van een faag therapeutische behandeling kan voorspellen. Dit model is gebaseerd op een eerder beschreven model van Hodyra-Stefaniak *et al.* (2015), beschreven in **Hoofdstuk 9**, waarbij we hun model verder hebben uitgebreid om rekening te houden met een anti-inflammatoire faag. Deze aanpassing voorspelt belangrijke gevolgen op de theoretische uitkomst van een faag therapeutische interventie. Ons model toont aan dat, wanneer de fagen anti-inflammatoire eigenschappen hebben, faag therapie succesvol is. Het initiële model van Hodyra-Stefaniak daarentegen toonde een falen van de faag therapeutische interventie voor het verwijderen van de bacteriële infectie aan.


ADDENDUM

Mathematical model

The original mathematical model described by Hodyra-Stefaniak *et al*. (2015), based on models of Levin and Bull (1996, 2004), and Payne and Jansen (2001, 2003) was expanded to include the anti-inflammatory properties of phages. The dynamics of bacteria, phage, innate and adaptive immunity and their interactions are described by differential equations. When the concentration of bacterial cells exceeds the threshold S_C and resources are unlimited, the concentration of innate system particles grows exponentially with a constant rate of a_I. When the phage concentration exceeds the threshold P_C and resources are unlimited, the concentration of the innate particles decrease at a constant rate of Υ_g, ultimately approaching one.

S: Growth of the bacterial population

$$\frac{dS(t)}{dt} = a_S S(t) - \rho S(t)P(t) - \kappa_{SI} S(t)I(t) - \kappa_{SB} S(t)B(t) \quad (1)$$

P: Dynamics of a free phage population

$$\frac{dP(t)}{dt} = b\rho S(t-\lambda)P(t-\lambda) - \rho S(t)P(t) - \kappa_{PI} P(t)I(t) - \kappa_{PA} P(t)A(t) \quad (2)$$

I: Innate immune response

$$\frac{dI}{dt} = \left(X_s(t) + X_P(t)\right)I(t) \quad (3)$$

X_S: Bacterial component of the innate immune response

$$X_S = \begin{cases} a_I \gamma_{gS}(t) \text{ for } S(t) \geq S_{C,} \\ -d_I \gamma_{dS}(t) \text{ otherwise} \end{cases} \quad (4)$$

Where

$$\gamma_{gS}(t) = \left(1 + \frac{S_C}{k_I}\right)\left(\frac{S(t)}{S(t) + k_I}\right) - \frac{S_c}{k_I}, \quad (5)$$

And

$$\gamma_{dS}(t) = \sqrt{1 - \frac{S(t)}{S_C}} \quad (6)$$

X_P: Phage component of the innate immune response

$$X_P = \begin{cases} -d_I \gamma_{dP}(t) \text{ for } P(t) \geq P_{C,} \\ a_I \gamma_{gP}(t) \text{ otherwise} \end{cases} \quad (7)$$

Where

$$\gamma_{gP}(t) = \left(1 + \frac{P_C}{k_I}\right)\left(\frac{P(t)}{P(t) + k_I}\right) - \frac{P_c}{k_I}, \quad (8)$$

And

$$\gamma_{dP}(t) = \sqrt{1 - \frac{P_C}{P(t)}} \quad (9)$$

A: Dynamics of adaptive immune response against phage

$$\frac{dA(t)}{d(t)} = a_A \left(\frac{P(t)}{P(t) + k_A}\right) A(t) \left(1 - \frac{A(t)}{A_{max}}\right) \quad (10)$$

B: Dynamics of adaptive immune response against bacteria

$$\frac{dB(t)}{d(t)} = a_B \left(\frac{S(t)}{S(t) + k_B}\right) B(t) \left(1 - \frac{B(t)}{B_{max}}\right) \quad (11)$$

Table A 1: State variables and parameters of the models described in Figure 9. 2.

State variables		Unit	Figure number								
			A	B	C	D	E	F	G	H	I
S	Concentration of susceptible bacteria	Cfu/ml	3×10^3	3×10^3	3×10^3	3×10^3	3×10^3	3×10^5	3×10^5	3×10^5	3×10^5
$P(t_P)$	Concentration of phages on time point p	Pfu/ml	2×10^5	2×10^5	2×10^5	2×10^8	2×10^8	2×10^4	2×10^4	2×10^5	2×10^5
I	Concentration of particles of the innate immune response	Particles/ ml	10^2	10^2	10^2	10^2	10^2	10^2	10^2	10^2	10^2
A	Concentration of particles of the adaptive immune response against phages	Particles/ ml	-	-	-	-	-	10^4	10^4	10	10
B	Concentration of particles of the adaptive immune response against bacteria	Particles/ ml	-	-	-	-	-	10^4	10^4	10^4	10^4
Parameters											
a_S	Growth rate of susceptible bacteria	h^{-1}	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7
a_I	Growth rate of innate immune response	h^{-1}	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5

a_A	Growth rate of adaptive immune response against phage	h^{-1}	-	-	-	-	-	0.9	0.9	0.9	0.9
a_B	Growth rate of adaptive immune response against bacteria	h^{-1}	-	-	-	-	-	0.9	0.9	0.9	0.9
d_I	Decay rate of innate immune response	h^{-1}	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
ρ	Adsorption rate of phages by susceptible bacteria	ml cfu^{-1} h^{-1}	10^{-8}	10^{-8}	10^{-8}	10^{-8}	10^{-8}	10^{-8}	10^{-8}	10^{-8}	10^{-8}
κ_{SI}	Killing rate of bacteria versus innate immune response	h^{-1}	10^{-6}	10^{-6}	10^{-6}	10^{-6}	10^{-6}	10^{-6}	10^{-6}	10^{-6}	10^{-6}
κ_{PI}	Killing rate of phages versus innate immune response	h^{-1}	0	10^{-3}	10^{-3}	10^{-3}	10^{-3}	10^{-3}	10^{-3}	10^{-3}	10^{-3}
κ_{SB}	Killing rate of bacteria versus adaptive immunity	h^{-1}	-	-	-	-	-	10^{-3}	10^{-3}	10^{-3}	10^{-3}
κ_{PA}	Killing rate of phages versus adaptive immunity	h^{-1}	-	-	-	-	-	10^{-3}	10^{-3}	10^{-3}	10^{-3}
S_C	Bacterial concentration above which innate immune response increases	Cfu/ml	100	100	100	100	100	100	100	100	100
P_C	Phage concentration above which innate immune response increases	Pfu/ml	-	10^5	-	-	10^5	-	10^5	-	10^5

Υ_g	Growth rate reduction of innate immune response		-	-	-	-	-	-	-	-
k_I	Bacterial concentration at which innate immune response actual growth rate is half of its maximum value	Cfu/ml	10^6	10^6	10^6	10^6	10^6	10^6	10^6	10^6
Υ_d	Decay rate reduction of innate immune response		-	-	-	-	-	-	-	-
b	Phage burst size	Pfu	40	40	40	40	40	40	40	40
λ	Latent period (average time between phage adsorption and burst	h	0.612	0.612	0.612	0.612	0.612	0.612	0.612	0.612
k_A	Phage concentration at which adaptive immune response actual growth rate is half of its maximum value	Pfu/ml	-	-	-	-	-	10^5	10^5	10^5
k_B	Bacterial concentration at which adaptive immune response actual growth rate is half of its maximum value	Cfu/ml	-	-	-	-	-	10^5	10^5	10^5
A_{max}	Maximum magnitude of A	Particles/ml	-	-	-	-	-	10^6	10^6	10^6
B_{max}	Maximum magnitude of B	Particles/ml	-	-	-	-	-	10^6	10^6	10^6

Table A 2: Differentially expressed genes exclusively present in the phage PNM stimulated dataset. Peripheral blood mononuclear cells (PBMCs) derived from one healthy individual were stimulated for 20 h with a *Pseudomonas aeruginosa* phage PNM lysate or its corresponding host (*P. aeruginosa* strain 573). Gene expression was normalized against a non-stimulated control condition. Differential gene expression between the *P. aeruginosa* phage PNM and *P. aeruginosa* strain 573 was compared to identify the genes uniquely expressed in the *P. aeruginosa* phage PNM stimulated dataset.

Gene symbol	log2Ratio (*P. aeruginosa* phage PNM/Control)	P-value	FDR
RMRP	-4.75	8.07E-06	1.07E-04
CAMK1	-4.15	5.69E-58	9.10E-56
FAM131C	-2.96	5.54E-24	2.83E-22
NCRNA00256 A	-2.56	4.85E-16	1.61E-14
NCRNA00256 B	-2.36	1.57E-10	3.60E-09
MEIS3	-1.79	2.68E-04	2.61E-03
SPRY2	-1.70	7.87E-10	1.72E-08
F13A1	-1.64	1.22E-08	2.39E-07
ADAMTSL4	-1.61	1.03E-08	2.04E-07
ALCAM	-1.57	1.95E-33	1.45E-31
CABP4	-1.44	2.42E-10	5.49E-09
ADAM28	-1.41	1.80E-22	8.34E-21
PCSK6	-1.40	5.67E-04	5.09E-03
HLA-DMA	-1.37	1.68E-28	1.05E-26
ADAMTS1	-1.30	1.04E-05	1.36E-04
LOC729041	-1.30	9.65E-04	8.13E-03
ITGA6	-1.30	1.96E-05	2.44E-04
NME1	-1.28	7.28E-12	1.87E-10
FLT3	-1.26	1.72E-04	1.74E-03
NCR3	-1.25	5.76E-13	1.57E-11
DENND1B	-1.24	1.09E-14	3.35E-13
NMUR1	-1.24	1.43E-06	2.13E-05
DHCR24	-1.21	6.55E-19	2.54E-17
LOC100505746	-1.21	3.36E-22	1.54E-20
PLCB1	-1.20	1.20E-04	1.26E-03
ZBED2	-1.16	2.10E-06	3.07E-05
KCP	-1.13	9.02E-04	7.66E-03

Gene symbol	log2Ratio (*P. aeruginosa* phage PNM/Control)	P-value	FDR
DGKE	-1.12	3.52E-11	8.56E-10
CTNNBIP1	-1.12	6.95E-08	1.25E-06
LY86	-1.11	7.98E-04	6.88E-03
GATM	-1.11	3.02E-05	3.64E-04
RAB37	-1.09	1.17E-13	3.38E-12
ITGAM	-1.08	1.94E-13	5.50E-12
HLA-DRB1	-1.07	9.18E-19	3.54E-17
PRSS23	-1.06	4.60E-06	6.30E-05
FCRL6	-1.05	1.62E-07	2.79E-06
LPAR6	-1.03	4.11E-15	1.30E-13
APOL4	-1.02	2.09E-05	2.58E-04
HLA-DMB	-1.02	1.29E-16	4.45E-15
DLEU1	-1.02	6.86E-04	6.04E-03
IER5	1.00	9.44E-20	3.82E-18
FMN1	1.01	1.73E-05	2.17E-04
LYN	1.01	4.19E-20	1.72E-18
LONRF3	1.01	5.22E-04	4.72E-03
STARD8	1.02	5.13E-10	1.14E-08
RNF169	1.03	8.89E-07	1.37E-05
LOC730227	1.03	2.54E-04	2.49E-03
ARHGAP31	1.03	2.91E-17	1.06E-15
NXPH4	1.03	7.45E-13	2.03E-11
CXCR6	1.03	1.08E-15	3.51E-14
MYO1G	1.03	4.43E-12	1.16E-10
LOC100133445	1.03	4.02E-14	1.20E-12
C5orf32	1.04	2.43E-15	7.74E-14
LCP2	1.04	7.36E-21	3.12E-19
NAB1	1.04	1.70E-11	4.26E-10
FAM167A	1.05	2.75E-06	3.91E-05
NFE2L2	1.05	1.10E-20	4.64E-19
VAC14	1.06	5.43E-20	2.21E-18
PRR16	1.06	5.55E-04	4.99E-03
NKD1	1.06	2.74E-08	5.13E-07

Gene symbol	log2Ratio (*P. aeruginosa* phage PNM/Control)	P-value	FDR
LRRC3	1.06	9.59E-05	1.03E-03
LILRB1	1.06	3.32E-05	3.96E-04
ACO1	1.06	8.37E-14	2.44E-12
LRP12	1.07	1.16E-07	2.03E-06
ERN1	1.07	4.01E-11	9.70E-10
CSNK1E	1.07	1.80E-18	6.87E-17
LYPD3	1.08	2.31E-11	5.70E-10
KCNG1	1.08	6.59E-04	5.83E-03
DLGAP3	1.08	3.29E-06	4.62E-05
MAP7	1.08	9.32E-04	7.88E-03
MTHFS	1.08	7.81E-07	1.22E-05
ZSWIM4	1.08	2.26E-14	6.79E-13
PMEPA1	1.09	2.76E-20	1.14E-18
SPTBN5	1.10	1.33E-05	1.70E-04
LRCH3	1.10	2.14E-06	3.12E-05
NINJ1	1.11	7.40E-24	3.77E-22
AQP9	1.11	1.26E-23	6.33E-22
LOC100131532	1.11	1.19E-03	9.75E-03
ELL	1.11	1.01E-22	4.81E-21
PPAP2B	1.11	1.88E-11	4.70E-10
SYN1	1.12	2.07E-11	5.15E-10
PNKD	1.12	5.85E-21	2.50E-19
RADIL	1.13	1.10E-03	9.13E-03
MAP4K4	1.13	1.68E-23	8.43E-22
HLX	1.13	1.57E-21	6.83E-20
LOC100130357	1.13	2.40E-06	3.45E-05
C6orf105	1.14	1.65E-11	4.15E-10
PPP4R2	1.14	8.58E-11	2.01E-09
CHST15	1.14	8.95E-23	4.27E-21
SCG5	1.15	1.28E-04	1.33E-03
TP53INP2	1.16	7.86E-23	3.77E-21
MGLL	1.16	1.15E-22	5.45E-21
PNPLA8	1.16	1.69E-08	3.23E-07

Gene symbol	log2Ratio (*P. aeruginosa* phage PNM/Control)	P-value	FDR
DENND5A	1.17	2.38E-25	1.31E-23
SPSB1	1.17	6.77E-15	2.11E-13
S100B	1.17	1.56E-04	1.59E-03
GPR157	1.17	5.31E-05	6.04E-04
OAF	1.18	7.49E-22	3.36E-20
CD48	1.18	1.07E-25	5.97E-24
SLFN5	1.18	3.96E-05	4.63E-04
C15orf48	1.19	6.66E-23	3.21E-21
TRIM56	1.19	1.49E-08	2.88E-07
RN7SL1	1.19	2.18E-04	2.16E-03
FLJ45445	1.19	5.40E-19	2.11E-17
PLK3	1.19	1.04E-25	5.81E-24
RABGEF1	1.20	7.83E-14	2.29E-12
FAM20A	1.20	4.73E-04	4.33E-03
TBC1D30	1.20	4.42E-13	1.22E-11
QPCT	1.21	3.79E-16	1.27E-14
FAM70B	1.21	1.45E-08	2.80E-07
AQP6	1.21	4.67E-05	5.40E-04
ABCA1	1.21	7.91E-25	4.23E-23
TNNT2	1.21	1.08E-05	1.40E-04
PANX1	1.21	1.09E-11	2.78E-10
LGALS3BP	1.21	1.03E-06	1.57E-05
RASD1	1.22	1.30E-07	2.26E-06
HELB	1.22	1.13E-04	1.19E-03
LOC650623	1.22	6.00E-05	6.75E-04
KIAA0247	1.22	5.80E-28	3.53E-26
SLC22A15	1.23	7.25E-16	2.38E-14
STX11	1.23	7.58E-26	4.27E-24
TNIP1	1.23	2.80E-29	1.82E-27
SESN2	1.24	1.17E-26	6.79E-25
SLC16A10	1.24	2.62E-24	1.37E-22
PTAFR	1.24	9.70E-09	1.93E-07
WBP5	1.25	6.96E-04	6.11E-03

Gene symbol	log2Ratio (*P. aeruginosa* phage PNM/Control)	P-value	FDR
A4GALT	1.26	4.83E-04	4.42E-03
XKR9	1.26	1.29E-04	1.35E-03
BTG3	1.26	1.72E-15	5.51E-14
DLC1	1.27	7.18E-04	6.27E-03
ABL2	1.27	1.10E-17	4.09E-16
ATP13A3	1.27	4.13E-30	2.74E-28
S1PR3	1.27	2.75E-04	2.67E-03
CDC42EP3	1.28	1.88E-22	8.70E-21
ELK1	1.28	1.37E-22	6.43E-21
CEP135	1.28	3.45E-23	1.70E-21
IRS2	1.28	3.35E-24	1.75E-22
NEFH	1.28	9.09E-04	7.70E-03
MAP3K4	1.29	8.50E-28	5.12E-26
ZP3	1.29	1.31E-08	2.56E-07
CSDA	1.30	6.64E-29	4.23E-27
NCRNA00241	1.30	3.28E-04	3.12E-03
SLC30A4	1.30	1.09E-08	2.14E-07
CFP	1.30	6.70E-06	8.98E-05
WLS	1.31	5.63E-06	7.64E-05
SPRED3	1.31	3.55E-06	4.95E-05
TJP2	1.32	6.96E-22	3.12E-20
FMNL2	1.33	2.08E-07	3.53E-06
MSC	1.33	7.11E-05	7.86E-04
TBC1D7	1.34	2.48E-17	9.04E-16
TMEM38B	1.35	4.93E-17	1.76E-15
DCUN1D3	1.35	6.20E-23	3.01E-21
PLAGL2	1.36	2.36E-12	6.27E-11
MN1	1.36	3.12E-22	1.43E-20
MB21D2	1.36	1.94E-18	7.37E-17
ANKRD57	1.37	1.84E-23	9.18E-22
SRC	1.37	3.32E-35	2.61E-33
TMEM44	1.38	2.79E-19	1.10E-17
L1CAM	1.38	1.60E-17	5.91E-16

Gene symbol	log2Ratio (*P. aeruginosa* phage PNM/Control)	P-value	FDR
ITIH1	1.39	5.18E-05	5.92E-04
RAB31	1.39	1.87E-34	1.44E-32
IRAK3	1.39	9.11E-30	6.03E-28
CREG1	1.39	1.72E-14	5.25E-13
MTF1	1.40	1.25E-33	9.38E-32
OSGIN2	1.41	2.13E-04	2.12E-03
ETS2	1.42	2.97E-18	1.13E-16
KNDC1	1.43	2.88E-05	3.49E-04
MEFV	1.43	6.06E-05	6.80E-04
C9orf46	1.43	1.17E-13	3.38E-12
SMPDL3A	1.43	2.51E-09	5.26E-08
LILRA1	1.44	2.87E-05	3.47E-04
GPR137B	1.44	3.01E-33	2.22E-31
ARL4A	1.45	4.80E-04	4.39E-03
TSPY26P	1.45	1.19E-04	1.25E-03
HYDIN2	1.46	5.33E-12	1.38E-10
MT1H	1.46	1.18E-03	9.70E-03
LAD1	1.47	8.86E-29	5.62E-27
ERGIC1	1.48	7.67E-38	6.77E-36
FLJ34208	1.48	8.45E-10	1.84E-08
ST6GALNAC2	1.49	7.13E-05	7.87E-04
FMO5	1.49	8.08E-07	1.26E-05
CLEC4D	1.50	1.84E-07	3.15E-06
FNDC3B	1.50	6.84E-32	4.81E-30
BASP1	1.50	1.78E-41	1.76E-39
ARHGEF10L	1.50	3.42E-35	2.68E-33
NANOS3	1.50	5.06E-04	4.60E-03
ATP1B4	1.50	4.50E-13	1.24E-11
PALM2-AKAP2	1.51	7.00E-08	1.26E-06
C12orf61	1.51	5.60E-07	8.97E-06
ALPK2	1.52	2.03E-12	5.40E-11
CPM	1.52	1.20E-15	3.87E-14

Gene symbol	log2Ratio (*P. aeruginosa* phage PNM/Control)	P-value	FDR
SPARC	1.52	4.84E-08	8.80E-07
CTSH	1.52	1.90E-42	1.99E-40
PLSCR1	1.53	5.16E-07	8.30E-06
LPP-AS2	1.53	1.35E-05	1.73E-04
HCK	1.54	4.62E-44	5.21E-42
CD82	1.56	1.63E-44	1.87E-42
UPB1	1.56	4.19E-09	8.66E-08
KCNH4	1.56	9.08E-07	1.40E-05
ENTPD7	1.58	1.01E-08	2.00E-07
MAFA	1.58	7.30E-04	6.36E-03
DFNA5	1.58	5.83E-16	1.93E-14
EBLN2	1.59	5.28E-04	4.77E-03
HTRA3	1.59	1.71E-04	1.73E-03
C9orf30	1.59	1.54E-33	1.15E-31
DLEU7	1.60	6.68E-05	7.42E-04
BAI1	1.60	1.67E-24	8.86E-23
DNER	1.60	2.37E-35	1.87E-33
CHI3L1	1.61	7.02E-04	6.16E-03
CH25H	1.61	1.01E-04	1.08E-03
PAPSS2	1.62	1.16E-16	3.99E-15
ARHGEF17	1.62	2.65E-11	6.52E-10
LY6E	1.63	1.66E-04	1.69E-03
SEMA3C	1.63	4.90E-15	1.54E-13
AMPD3	1.63	1.33E-45	1.58E-43
WNK2	1.64	1.09E-07	1.92E-06
TRAF3IP2	1.64	8.42E-43	9.06E-41
VNN3	1.66	5.91E-21	2.51E-19
LOC200261	1.66	2.52E-08	4.73E-07
MT1F	1.67	1.18E-16	4.08E-15
HRH1	1.67	1.82E-17	6.68E-16
IRF7	1.67	3.59E-07	5.92E-06
AGRN	1.69	2.88E-31	2.00E-29
CCRL2	1.69	5.83E-46	7.09E-44

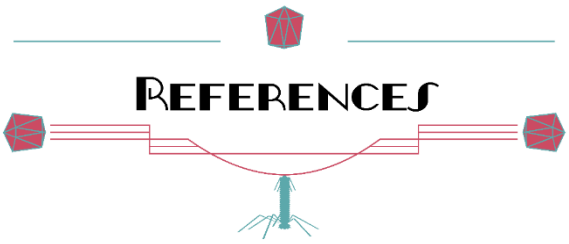
Gene symbol	log2Ratio (*P. aeruginosa* phage PNM/Control)	P-value	FDR
SLCO4A1	1.71	8.32E-04	7.15E-03
SMTN	1.72	1.69E-10	3.85E-09
NAMPT	1.73	2.29E-54	3.35E-52
KANK1	1.74	6.00E-24	3.06E-22
SLC2A6	1.75	7.59E-54	1.10E-51
TGFA	1.76	1.05E-12	2.83E-11
HMGN2P46	1.77	1.25E-12	3.37E-11
ZC3H12A	1.77	3.24E-29	2.10E-27
NLRP3	1.78	4.04E-24	2.08E-22
ADA	1.78	5.21E-55	7.68E-53
EHD1	1.79	1.08E-57	1.69E-55
MYLK	1.80	5.33E-05	6.07E-04
LDLRAD3	1.83	4.35E-11	1.05E-09
CDC42EP2	1.86	4.06E-58	6.56E-56
MCTP1	1.86	1.68E-07	2.89E-06
TBC1D9	1.87	4.96E-49	6.50E-47
ZFP92	1.88	5.92E-04	5.28E-03
P2RY2	1.89	3.97E-07	6.50E-06
TMEM54	1.90	1.31E-05	1.68E-04
ZBED3	1.92	3.79E-04	3.54E-03
NTSR1	1.93	1.37E-12	3.65E-11
ACSL5	1.94	3.16E-16	1.06E-14
TM4SF19	1.94	1.11E-15	3.60E-14
SLC24A4	1.94	4.12E-16	1.38E-14
MPZL1	1.96	5.48E-23	2.67E-21
NKX3-1	1.96	5.82E-16	1.92E-14
C13orf29	1.98	2.41E-06	3.47E-05
C22orf45	2.00	2.96E-09	6.16E-08
NR6A1	2.00	2.81E-04	2.72E-03
SCARF1	2.02	9.46E-22	4.20E-20
SOCS3	2.02	2.57E-44	2.93E-42
SPATC1	2.03	2.16E-09	4.56E-08
PHACTR1	2.06	2.24E-23	1.12E-21

Gene symbol	log2Ratio (*P. aeruginosa* phage PNM/Control)	P-value	FDR
TBC1D16	2.06	9.10E-07	1.40E-05
LIF	2.10	3.79E-46	4.67E-44
PTGIR	2.12	3.67E-52	5.09E-50
HRH2	2.13	2.37E-05	2.90E-04
LOC100127888	2.15	5.14E-04	4.66E-03
EIF2AK2	2.16	3.69E-05	4.36E-04
FNIP2	2.16	2.79E-57	4.31E-55
RFX8	2.19	1.21E-10	2.80E-09
SOD2	2.21	6.80E-22	3.06E-20
DTX4	2.21	1.52E-05	1.92E-04
STON2	2.21	1.27E-04	1.33E-03
MT1G	2.21	3.15E-08	5.85E-07
STEAP1	2.22	7.91E-06	1.05E-04
LIMK2	2.23	1.14E-83	3.04E-81
DUSP1	2.25	4.59E-29	2.94E-27
LILRA3	2.29	2.23E-05	2.74E-04
C1orf61	2.29	9.93E-05	1.06E-03
FFAR2	2.31	1.23E-06	1.87E-05
BCAT1	2.31	9.30E-80	2.20E-77
MARCKS	2.34	2.39E-92	7.84E-90
SIGLEC14	2.34	1.51E-18	5.80E-17
CASP5	2.35	2.17E-20	8.99E-19
MT2A	2.35	1.46E-84	3.96E-82
STC2	2.35	8.13E-13	2.20E-11
ARNT2	2.37	2.06E-65	3.81E-63
CXCL3	2.37	1.07E-27	6.41E-26
TRIM36	2.37	1.43E-15	4.61E-14
HS3ST3B1	2.37	7.38E-58	1.17E-55
C19orf59	2.38	2.32E-90	7.24E-88
DCBLD1	2.41	7.16E-21	3.04E-19
HBEGF	2.41	1.24E-91	4.00E-89
MT1E	2.41	1.79E-20	7.43E-19
CLGN	2.42	1.05E-04	1.12E-03

Gene symbol	log2Ratio (*P. aeruginosa* phage PNM/Control)	P-value	FDR
SERPINB2	2.43	3.42E-08	6.32E-07
NPHS1	2.45	1.44E-04	1.48E-03
SERPINA1	2.46	6.53E-103	2.78E-100
ACSL1	2.47	2.14E-98	8.00E-96
FAM110B	2.54	1.06E-15	3.46E-14
ADAMTS14	2.55	1.03E-47	1.31E-45
CDK1	2.55	4.75E-14	1.41E-12
GPR84	2.56	1.47E-82	3.76E-80
PLAUR	2.56	4.00E-78	9.02E-76
WNT5A	2.58	2.84E-23	1.40E-21
CACNA2D3	2.58	6.61E-06	8.87E-05
SIAH3	2.59	2.93E-06	4.15E-05
OSMR	2.59	9.60E-07	1.47E-05
FAM108C1	2.60	8.78E-36	7.06E-34
KRT86	2.64	4.89E-25	2.63E-23
MMP14	2.65	7.41E-118	3.86E-115
CLEC4E	2.68	6.06E-89	1.77E-86
CCNE2	2.70	1.21E-05	1.55E-04
GLIS3	2.73	7.83E-36	6.34E-34
GUCY1B2	2.74	9.81E-09	1.95E-07
LRRC38	2.78	5.26E-08	9.54E-07
BMP6	2.85	3.30E-18	1.25E-16
PAPLN	2.89	2.40E-47	3.02E-45
SIGLEC5	2.90	3.25E-37	2.83E-35
SLC9A7P1	2.93	1.42E-19	5.70E-18
MT1M	2.95	7.66E-15	2.37E-13
KL	2.96	1.50E-06	2.24E-05
CXCL1	2.99	1.98E-11	4.94E-10
SLC7A7	3.01	3.92E-58	6.39E-56
CCL23	3.01	7.90E-07	1.23E-05
TRPM2	3.03	3.04E-135	2.19E-132

Gene symbol	log2Ratio (*P. aeruginosa* phage PNM/Control)	P-value	FDR
CXCL6	3.09	1.16E-05	1.50E-04
LOC100652730	3.10	4.19E-08	7.69E-07
SGIP1	3.19	3.45E-05	4.10E-04
TRPV4	3.21	3.28E-04	3.12E-03
FJX1	3.22	1.44E-13	4.12E-12
GJA1	3.26	1.36E-04	1.40E-03
CES1	3.31	1.70E-24	8.98E-23
GPR124	3.35	3.22E-11	7.83E-10
PNPLA1	3.36	2.14E-39	1.99E-37
FGF2	3.37	1.68E-08	3.22E-07
LARP6	3.39	4.11E-29	2.65E-27
STEAP3	3.41	1.12E-08	2.19E-07
KREMEN1	3.43	3.43E-162	3.21E-159
TDRD9	3.43	1.10E-27	6.55E-26
ZDHHC19	3.51	3.48E-06	4.86E-05
FAM124A	3.54	1.21E-38	1.10E-36
SERPIND1	3.58	1.46E-10	3.37E-09
SH3PXD2B	3.61	5.82E-35	4.52E-33
IGFN1	3.68	3.20E-122	2.00E-119
C3	3.69	3.89E-37	3.37E-35
DYSF	3.72	1.82E-34	1.40E-32
SBSN	3.90	1.73E-07	2.96E-06
SLC24A3	3.92	2.11E-14	6.37E-13
HAS1	4.00	6.37E-10	1.40E-08
TMEM132A	4.09	4.62E-17	1.66E-15
ITGB3	4.26	2.73E-141	2.13E-138
TNFRSF6B	4.41	4.39E-43	4.78E-41
DDAH1	4.43	1.40E-04	1.45E-03
C9orf70	4.58	3.88E-05	4.56E-04
RETN	4.72	6.23E-23	3.01E-21
ATOH8	4.78	2.41E-07	4.08E-06

Gene symbol	log2Ratio (*P. aeruginosa* phage PNM/Control)	P-value	FDR
TBX3	4.95	3.11E-07	5.16E-06
C20orf160	5.17	6.26E-165	6.51E-162
IL36B	6.02	6.68E-14	1.96E-12
CA12	6.34	2.19E-06	3.17E-05
FLJ36644	12.99	1.10E-03	9.10E-03
CDC42EP5	13.41	7.10E-04	6.22E-03
LOC100507410	13.58	3.02E-05	3.63E-04



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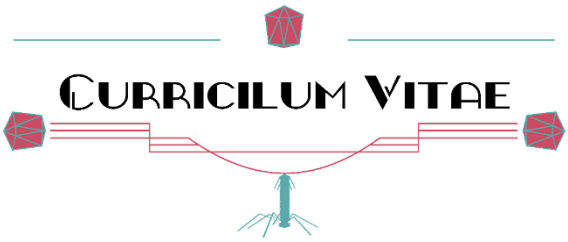
Curricilum Vitae

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EDUCATION

01/2014 – 12/2017	PhD student *Immunomodulation by Bacteriophages.* Laboratory Bacteriology Research Department Clinical Chemistry, Microbiology and Immunology Faculty of Medicine and Health Sciences, Ghent University Promoter: Prof. Dr. Mario Vaneechoutte Co-promoter: Prof. Dr. Rob Lavigne (KULeuven)
09/2011 – 07/2013	Master Biochemistry-Biotechnology, Ghent University Master thesis: Study of *Staphylococcus aureus* phage ISP and derived proteins. Potential use as antibacterial therapeutics and anti-inflammatory properties Promoter: Prof. Dr. Mario Vaneechoutte *Great distinction*
09/2008 – 07/2011	Bachelor Biochemistry-Biotechnology, Ghent University *Distinction*
09/2002 – 07/2008	Humaniora Broeders, Sint-Niklaas
09/1994 – 07/2002	Basisschool Broeders, Sint-Niklaas

PUBLICATIONS

4. **Van Belleghem, J.D.**, Merabishvili, M., Clement, F., Lavigne, R. & Vaneechoutte, M. Pro- and anti-inflammatory responses of peripheral blood mononuclear cells induced by *Staphylococcus aureus* and *Pseudomonas aeruginosa* phages. *Sci. Rep.* **8**: 8004 – 8016 (2017)
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3. Merabishvili, M., Monserez, R., **Van Belleghem, J.D.**, Rose, T., Jennes, S., De Vos, D., Verbeken, G., Vaneechoutte, M. & Pirnay, J.P. Stability of bacteriophages in burn wound care products. *Plos One* **12**(7):e0182121 (2017)

2. **Van Belleghem, J.D.**, Merabishvili, M., Vergauwen, B., Lavigne, R. & Vaneechoutte, M. A comparative study of different strategies for removal of endotoxins from bacteriophage preparations. *J. Microbiol. Methods* **132**, 153 – 159 (2017)

1. Van Hoecke, H., De Paepe, A., Lambert, E., **Van Belleghem, J.D.**, Cools, P., Van Simaey, L., Deschaght, P., Vaneechoutte, M. & Dhooge, I. Haemophilus influenzae biofilm formation in chronic otitis media with effusion. *Eur. Arch. Oto-Rhino-Laryngol.* **273**, 3553 – 3560 (2016)

CONFERENCES/SYMPOSIA/WORKSHOPS

06/08/2017 – 11/08/2017: 22st Biennial Evergreen International Phage Meeting
Presentation:
Effects of bacteriophages on the human immune response
The Evergreen State College, Olympia, Washington

26/06/2017 – 29/06/2017: Centennial Celebration of Bacteriophage Research
Presentation:
Immunomodulation by bacteriophages
Tbilisi, Georgia

17/01/2017 – 19/07/2017: Bacteriophage 2017
Attendant
Online

18/07/2016 – 22/07/2016: Viruses of microbes 2016
Poster presentation:
Immunomodulation by bacteriophages - Differential expression of immune cells stimulated with different phages
Liverpool, United Kingdom

20/10/2015: Training at VIB: symposium 'Emerging Cytokine Networks'
Attendant
Ghent, Belgium

29/09/2015: Phages in interaction IV
Presentation + Poster presentation:
Immunomodulation by bacteriophages - Differential expression of immune cells stimulated with different phages
Leuven (Heverlee), Belgium

02/08/2015 – 07/08/2015: 21st Biennial Evergreen International Phage Meeting
Poster presentation:
Immunomodulation by bacteriophages - Differential expression of immune cells stimulated with a *Pseudomonas aeruginosa* phage
The Evergreen State College, Olympia, Washington

15/06/2015 – 19/06/2015:	2nd International Hands-on Phage Biotechnology course Attendant Braga, Portugal
17/03/2015 – 18/03/2015:	Bacteriophages in the 21st Century – Diversity and Potential Applications Invited speaker (Phages and the Immune System) Scientific Symposium of the Leibniz-Institute DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen. Braunschweig, Germany
08/09/2014 – 12/09/2014:	Flanders Training Network for Methodology and Statistics (FLAMES) summer school Attendant Ghent, Belgium
14/07/2014 – 18/07/2014:	Viruses of microbes: Structure and function, from molecules to communities Attendant Zurich, Switzerland.

GRANTS/FUNDS/SCHOLARSHIPS

01/01/2014 – 01/01/2018:	IWT/FWO: PhD Scholarship Strategic Basic Research