– Acts of Give and Take –
The multifactorial regulation of the immune checkpoint PD-L1 in the course of *H. pylori* infection

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Dedication

to Domino,

my fluffy shadow, who forces me to go outside for walks and
devote my time to him so he won't turn into a disobeying brat
as of 2008, when my path in biology started

Selbstständigkeitserklärung

Hiermit erkläre ich, Janine Sigulla, geboren am 19.06.1989 in Grimma, die Dissertation selbstständig und nur unter Verwendung der angegebenen Hilfen und Hilfsmittel angefertigt zu haben. Ich habe mich anderwärts nicht um einen Doktorgrad beworben und besitze keinen entsprechenden Doktorgrad. Ich erkläre, dass die Dissertation oder Teile davon nicht bereits bei einer anderen wissenschaftlichen Einrichtung eingereicht, angenommen oder abgelehnt wurden. Ich erkläre die Kenntnisnahme der dem Verfahren zugrunde liegenden Promotionsordnung der Lebenswissenschaftlichen Fakultät der Humboldt-Universität zu Berlin vom 5. März 2015. Weiterhin erkläre ich, dass keine Zusammenarbeit mit gewerblichen Promotionsberatern stattgefunden hat und dass die Grundsätze der Humboldt-Universität zu Berlin zur Sicherung guter wissenschaftlicher Praxis eingehalten wurden.

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Janine Sigulla

List of Publications

Doctoral Thesis research

in preparation **Sigulla**, Pfannkuch, Müller, Al-Zeer, Meyer: Multifactorial regulation of PD-L1 in the course of *Helicobacter pylori* infection

2019 Pfannkuch, Hurwitz, Traulsen, **Sigulla**, Poeschke, Matzner, Kosma, Schmid, Meyer: ADP heptose, a novel pathogen-associated molecular pattern identified in Helicobacter pylori. FASEB J. 2019 Aug;33(8):9087-9099. Doi: 10.1096/fj.201802555R. Epub 2019 Jun 21.

2017 Al-Zeer, Xavier, Abu Lubad, **Sigulla**, Kessler, Hurwitz, Meyer: Chlamydia trachomatis Prevents Apoptosis Via Activation of PDPK1-MYC and Enhanced Mitochondrial Binding of Hexokinase II. EBio Medicine. 2017 Sep; 23:100-110. doi: 10.1016/j.ebiom.2017.08.005. Epub 2017 Aug 5

Master Thesis research

2017 Touat-Todeschini, Shichino, Dangin, Thierry-Mieg, Gilquin, Hiriart, Sachidanandam, Lambert, **Brettschneider**, Reuter, Kadlec, Pillai, Yamashita, Yamamoto, Verdel: Selective termination of lncRNA transcription promotes heterochromatin silencing and cell differentiation. EMBO J. 2017 Sep 1;36(17):2626-2641. doi: 10.15252/embj. 201796571. Epub 2017 Aug 1.

2014 Dias, Van Nguyen, Georgiev, Gaub, **Brettschneider**, Cusack, Kadlec, Akhtar: Structural analysis of the KANSL1/WDR5/KANSL2 complex reveals that WDR5 is required for efficient assembly and chromatin targeting of the NSL complex. Genes Dev. 2014 May 1;28(9):929-42. doi: 10.1101/gad.240200.114.

Zusammenfassung

Hintergrund: Viren und Bakterien stellen eine ständige Herausforderung für unsere Immunität und selbst unsere Gesellschaft dar. Während bestimmte Krankheitserreger durch ein funktionierendes Immunsystem beseitigt werden können, können andere ein Leben lang im menschlichen Körper verbleiben. Eines der erfolgreichsten Bakterien ist *Helicobacter pylori*, das in ca. der Hälfte der Weltbevölkerung persistiert (Parsonnet 1995). Ausgestattet mit Virulenzfaktoren kann der Erreger das Magenepithel erreichen. Dort wird die frühe Erkennung von Bakterien und die anschließende Entzündung über den Zucker Heptose-1,7-bisphosphat (HBP) ausgelöst, einem Zwischenprodukt des ADP-Heptose-Synthesewegs, welches für LPS, einer Zellwandkomponente der meisten Gram-negativen Bakterien, benötigt wird (Zimmermann, Pfannkuch et al. 2017). Da dies jedoch normalerweise nicht ausreicht, um die Infektion zu beseitigen, geht die bakterielle Persistenz mit einer chronischen Gastritis einher, die bis zu Magenkrebs fortschreiten kann (Correa, Haenszel et al. 1975). Es wurde gezeigt, dass eine streng regulierte T-Zellpopulation ursächlich für den Schutz der Wirtszellen und der gleichzeitigen Toleranz gegenüber *H. pylori* ist (Ermak, Giannasca et al. 1998). Immunzellregulation, wie der Ligand PD-L1 spielen eine wichtige Rolle bei der Persistenz von Infektionen und der Entstehung von Krebs. Es wurde gezeigt, dass Magenepithelzellen auf eine *H. pylori*-Infektion mit einer erhöhten PD-L1-Expression reagieren (Das, Suarez et al. 2006). Diese Studie zielte darauf ab, das Wissen um die Wirkmechanismen zu erweitern, die dem *H. pylori*-vermittelten PD-L1-Induktionsprozess zugrunde liegen.

Methoden: Zeitpunkte, in denen der Ligand hochreguliert wurde, wurden individuell analysiert und zwei Ansätze wurden verfolgt:

I. Eine chromatographische Analyse wurde genutzt, um auf aktivierende Faktoren zu prüfen. In diesem Zusammenhang wurden Derivatisierungen, enzymatische Verdau-Prozesse, Reporter- als auch Knockout-Zelllinien und chemisch synthetisierte Verbindungen verwendet. Weitere Implikationen wurden mittels einer Microarray-Analyse untersucht.

II. Microarray-Daten wurden genutzt, um die Infektion mit den Bakterium zu charakterisieren, denen der identifizierte PD-L1-regulierende Faktor fehlt. Inhibitionsversuche, siRNA-vermittelte Knockdowns und die Erzeugung einer CRISPR-Cas9-Knockout-Zelllinie wurden zur Validierung genutzt.

Ergebnisse: Die Regulation von PD-L1 ist ein komplexer Prozess. Es wurden zwei unterschiedliche und unabhängig- ursächliche Mechanismen identifiziert.
Induktionsphase (I) hängt von der Existenz des bakteriellen Sekretionssystems cagT4SS und eines funktionierenden ADP-Heptose-Synthesewegs ab. Durch Kombination von massenspektrometrischen Analysen mit chemischen und enzymatischen Behandlungen wurde ADP-Heptose als Inflammationsauslöser gezeigt. Weder der Vorläufer HBP noch das Endprodukt LPS waren in der Lage, PD-L1 zu induzieren. Allein ADP-Heptose-behandelte Zellen induzierten den Liganden schnell. Eine Microarray-Analyse lieferte Hinweise darauf, dass ADP-Heptose ausschließlich von der Wirtskinase ALPK1 erkannt wird, wobei die Signalachse ein Haupttreiber für verschiedene Signalwege ist, einschließlich Hypoxie und der Epithelial-mesenchymalen Transition.
Im Gegensatz dazu wurde festgestellt, dass die PD-L1-Induktionsphase (II) auf der metabolischen Reprogrammierung des Wirts beruht. Ein Merkmal von *H. pylori* ist dessen Bedarf an Cholesterin, welches aus dem Medium oder aus membranösen Lipidregionen des Wirts extrahiert wird. Es konnte bewiesen werden, dass dieser Sterol-Abbauprozess zu einer erhöhten Stoffwechselaktivität führt, die spezifisch mit einer Zunahme der Glykolyse verbunden ist und mit einer Expressionsverschiebung des ersten Glykolyseenzyms Hexokinase 1 zu Hexokinase 2 einhergeht. Es wurde gezeigt, dass die Induktion des Immunliganden PD-L1 von der aberranten HK2-Expression abhängt.
Mit den erzeugten Ergebnissen wurden neue Erkenntnisse über das multifaktorielle Regulationsnetzwerk von PD-L1 während einer *H. pylori*-Infektion gewonnen: Es wurde ein neuer bakterieller Metabolit, der eine starke Entzündungsreaktion hervorrufen kann, identifiziert und erstmals wurde gezeigt, dass eine metabolische Reprogrammierung, die mit einer Expressionsverschiebung zugunsten HK2s einhergeht, PD-L1 reguliert.

Summary

Background: Viruses and bacteria represent a constant challenge to our immune integrity and even society. While certain pathogens are able to get eradicated by a functioning immune system, others are capable to persist in the human body over a lifetime. One of the most successful bacterium is *Helicobacter pylori*, found in about half of the worlds' population (Parsonnet 1995). Equipped with virulence factors, the pathogen is able to reach the gastric epithelium. There, early bacterial recognition and subsequent inflammation is triggered via the sugar heptose 1,7-bisphosphate (HBP), an intermediate of the ADP heptose synthesis pathway, needed for LPS, a cell wall component of most Gram-negative bacteria (Zimmermann, Pfannkuch et al. 2017). However, as this is usually not sufficient to clear infection, bacterial persistency is accompanied by chronic gastritis which can progress over atrophic gastritis towards gastric cancer.(Correa, Haenszel et al. 1975) A tightly regulated T cell population was shown to be majorly responsible for host cell protection, while maintaining tolerance towards *H. pylori* (Ermak, Giannasca et al. 1998). Immune cell regulators, like programmed death ligand 1 (PD L1), play an important role in the persistency of infection and the establishment of cancer. It has been shown that gastric epithelial cells respond to *H. pylori* infection with an increased PD-L1 expression (Das, Suarez et al. 2006). This study aimed to broaden this knowledge and decipher mechanism underlying the *H. pylori*–mediated PD-L1 induction process.

Methods: Upregulation incidences were individually analyzed and in an attempt to deepen the understanding of participating pathways, two approaches were applied:
I. Chromatographic analysis were used to check for activating factors. In this context, derivatization, enzymatic digestion, reporter or knockout cell lines and chemical synthesized compounds were used. Implications of newly identified bacterial factors in the infection context were gained by performing microarray analysis.
II. Previously existing microarray data was used to characterize infection with bacteria, lacking the identified PD L1 regulating factor. In order to validate the

findings, a set of techniques was used, including chemical treatments, siRNA-mediated knockdowns and the generation of a CRISPR-Cas9 knockout cell line.

Results: Regulation of PD-L1 is a complex process. Two distinct and independent causative mechanism were identified.

Induction phase (I) was shown to depend on the existence of the bacterial secretion system cagT4SS and a functioning ADP heptose synthesis pathway. By combining mass spectrometric analysis with chemical and enzymatical treatments, evidence for ADP heptose as an immune stimulator was proven. Neither the precursor HBP, nor the final product LPS, were capable to induce PD-L1. Only ADP heptose-challenged cells induced the ligand rapidly. Additionally, it was the only compound able to provoke an inflammatory response, when added extracellularly. A microarray analysis provided evidence that ADP heptose is exclusively sensed by the host kinase alpha-kinase 1 (ALPK1), with the signaling axis being a major driver for several pathways, including hypoxia and epithelial-mesenchymal transition.

By contrast, PD-L1 induction phase (II) was identified to rely on metabolic reprogramming of the host. A characteristic of *H. pylori* is the need for cholesterol, which it either extracts from the medium or from membranous lipid regions of the host. Within this work, it could be demonstrated, that the sterol depletion process results in enhanced metabolic activity which is specifically linked with an increase in glycolysis and accompanied by expression shift of the first glycolysis enzyme hexokinase 1 to hexokinase 2. Using knockdown and knockout experiments, it was noted that induction of the immune ligand PD-L1 depends on the aberrant HK2 expression.

With the obtained results, new insights in the multifactorial regulation network of PD-L1 during *H. pylori* infection was gained: A new bacterial metabolite, capable to elicit strong inflammatory response, was identified and, for the first time, metabolic reprogramming with expression shift to HK2 was demonstrated to regulate PD L1.

Table of contents

List of figures

List of tables

Abbreviations

%	percentage
µg	microgram
µL	microliter
µm	micrometer
µM	micromolar
2D	two dimensional
AEC	3-amino-9-ethylcarbazole
ADP	adenosine diphosphate
ADP	adenosine diphosphate
AGS	human epithelial gastric adenocarcinoma cell line
ALPK1	alpha-kinase 1
APC	antigen presenting cell
BAFF-R	B-cell activating factor receptor
CagA	cytotoxin-associated gene A
cagPAI	cytotoxin-associated gene pathogenicity island
Cas9	CRISPR associated protein 9
CD	cluster of differentiation
CFU	colony forming units
CIP	alkaline phosphatase
CLR	C-type lectin receptor
CO_2	carbonate dioxide
CRISPR	clustered regularly interspaced short palindromic repeats
CTL	clotrimazol
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
DC	dendritic cell
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP's	dioxynucleotide triphosphates
DPBS	Dulbecco's phosphate buffered saline
DSB	double-strand break
DTT	dithiotreitol
EDTA	ethylenediaminetetraacetic acid
ESI	electrospray ionization
EtBr	ethidium bromide
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
g	gramm
GAPDH	glyceraldehyde-3-phosphate dihydrogenase
GSEA	gene set enrichment analysis
H. pylori	*Helicobacter pylori*
HBP	heptose 1,7-bisphosphate
HK	Hexokinase
HopQ	Helicobacter outer membrane protein Q
hpi	hours post infection
IFN	interferon
Ig	immunoglobulin
IKK	IκB kinase
IL	interleukin
irAEs	immune-related adverse events (irAEs)
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibition motif
ITRM	immunoreceptor tyrosine-based regulation motif
ITSM	immunoreceptor tyrosine-based switch motif
IκB	inhibitor of kappa B
kb	kilobase pair
KD	knockdown
kDa	kilodalton
Kdo	3-deoxy-D-*manno*-oct-2-ulosonic acid or keto-deoxyoctulosonate
KO	knockout
LC-MS/MS	liquid chromatography-mass spectrometry/mass spectrometry
Log	logarithm
LPS	lipopolysaccharide
LTβR	lymphotoxin β receptor
m/z	mass to charge ratio
MALT	mucosa-associated lymphoid tissue
mBCD	methyl-β-cyclodextrin
MHC	major histocompatibility complex
ml	milliliter
mM	millimolar
MOI	multiplicity of infection
MPIIB	Max Planck Institute for Infectionbiology
mRNA	messenger ribonucleic acid
MS	mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MyD88	myeloid differentiation primary response gene 88

NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NHEJ	non-homologous end joining
NI	Non-infected
NIC	nicotinamide
NLR	NOD-like receptor
NLRP3	NLR family pyrin domain containing 3
NOD	nucleotide-binding oligomerization domain
NSCLC	non-small-cell lung carcinoma
NT	non-treated
OD	optical density
P12Δ	*H. pylori* strain P12 deletion mutant
PAGE	polyacrylamide gel electrophoresis
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD-1	programmed cell death protein 1
PD-L1	programmed death-ligand 1
PFA	paraformaldehyde
pH	potential of hydrogen
PRR	pattern recognition receptor
RANK	receptor activator of NF-κB
RelA	v-rel avian reticuloendotheliosis viral oncogene homolog A
RIG	retinoic acid inducible gene
RIP2	receptor-interacting serine/threonine kinase 2
RLR	RIG-I-like receptor
RNA	ribonucleic acid
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	revolutions per minute
RSPO	R-spondin 1
RT	room temperature or reverse transcriptase
RT-qPCR	reverse transcription quantitative real-time PCR
SD	standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH	Src-homology
NAD(P)H	nicotinamide adenine dinucleotide (phosphate)
SHP	SH domain-containing tyrosine phosphatase
SYK	spleen tyrosine kinase
T	threonine residue
T4SS	type IV secretion system
TFA	trifluoroacetic acid
TAB	TAK1-binding protein
TAK1	transforming growth factor (TGF)-β-activated kinase 1
Taq	*Thermus aquaticus*
TBE	Tris-Borate EDTA
TBS	Tris-buffered saline (with Tween20)
TBS-T	Tris-buffered saline containing Tween20
TIFA	TRAF-interacting protein with forkhead-associated domain
TLR	Toll-like receptor
TNFα	tumor necrosis factor (alpha)
TRAF	TNF receptor associated factor
Treg	regulatory T-cell
Tris	tris(hydroxymethyl)aminometha
UPLC	Ultra-Performance Liquid Chromatography
UV	ultra violet
VacA	vacuolating toxin A
vs	versus
w/v	weight per volume
WB	western blot
Wnt	Wingless-Type MMTV Integration Site
wt	wildtype
Y	tyrosine residue

Chapter

1. Introduction

The mammalian immune system and immune evasion by *Helicobacter pylori*

Our immune integrity is constantly challenged by our surrounding. Pathogenic microorganisms, toxic or allergenic proteins and abnormal cells need to be discriminated from beneficial commensal microbes, nutrients and healthy cells. A balanced response to control or eliminate threats by simultaneously avoiding the destruction of microbiota or excessive damage of healthy tissue is indispensable. Distinguished by speed and specificity, the immune system in higher animals is divided into *adaptive* and *innate* immune response. The rapid innate immune response is conserved in all multicellular organisms and acts as a first layer of defense. If required, the adaptive immune system joins for help. Both systems rely on different mechanism (Figure 1.1): The innate immune system is based on sensor proteins (PRRs), that recognize specific molecule patterns which are common among pathogens but absent in the host. For example, in Gram-negative bacteria the integral metabolite of the LPS synthesis pathway, HBP was shown to act as such a factor triggering inflammatory response by activation of the kinase ALPK1. In contrast, the adaptive immune system uses a more sophisticated mechanism through which it produces, by genetic recombination, an almost limitless diversity of receptors on T and B cells and secreted antibodies to detect foreign molecules. Naïve immune cells are recruited by innate immune cells and mediator substances to the site of infection or mature through various stimuli towards effector and memory cells. These processes are tightly controlled and one of the regulators is the surface protein PD-L1. It is a ligand for the co-signaling receptor PD-1 and a well-studied inhibitor of lymphocyte function. The PD-L1:PD-1 axis plays a central role in lymphocyte development, establishment of immunological tolerance and immune exhaustion, a hallmark of cancer. Therefore, in the last decade it became of interest as a target for cancer therapy and together with the co-signaling receptor CTL-4 and its ligands, PD-L1:PD-1 inhibition started the branch of immune checkpoint therapy with promising results. In the context of infection, PD-L1 was shown to be crucial to balance effective antimicrobial defense and inflammation-associated tissue damage. A challenge to immune integrity is represented by the gastric Gram-negative bacterium *Helicobacter pylori* which colonizes

approximately half of the world's population. It is well adapted to co-exist in the human stomach for a life-time and developed sophisticated methods to avoid immune recognition and subsequent clearance. Infection leads to gastritis which can, depending on bacterial and host factors, proceed towards tissue ulceration, lymphoma development or gastric cancer. Therefore, as the only bacterium so far, *H. pylori* was classified as a class I carcinogen and it is of major interest to understand its immune evasion process.

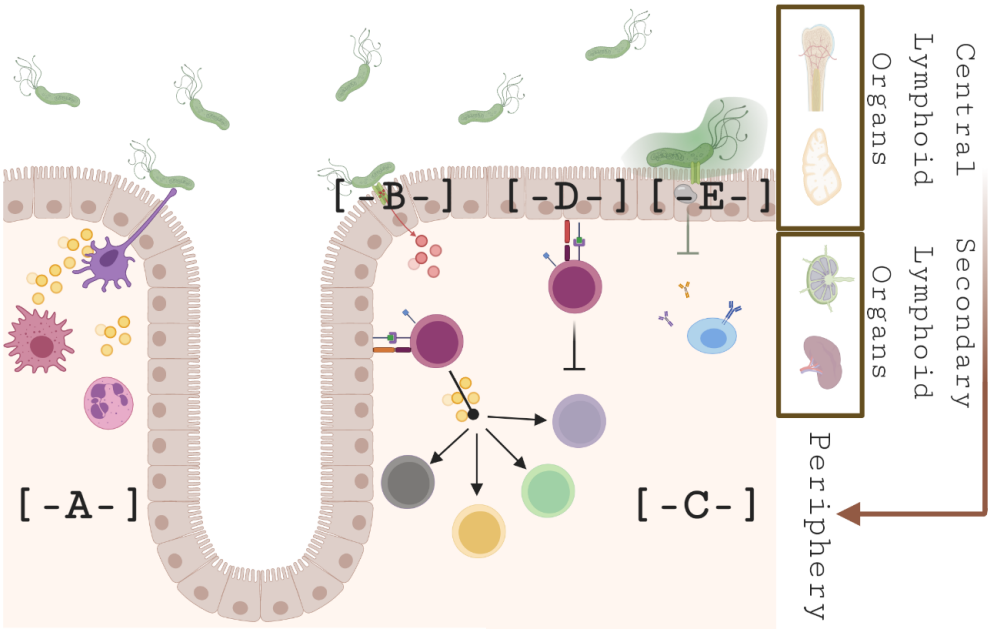


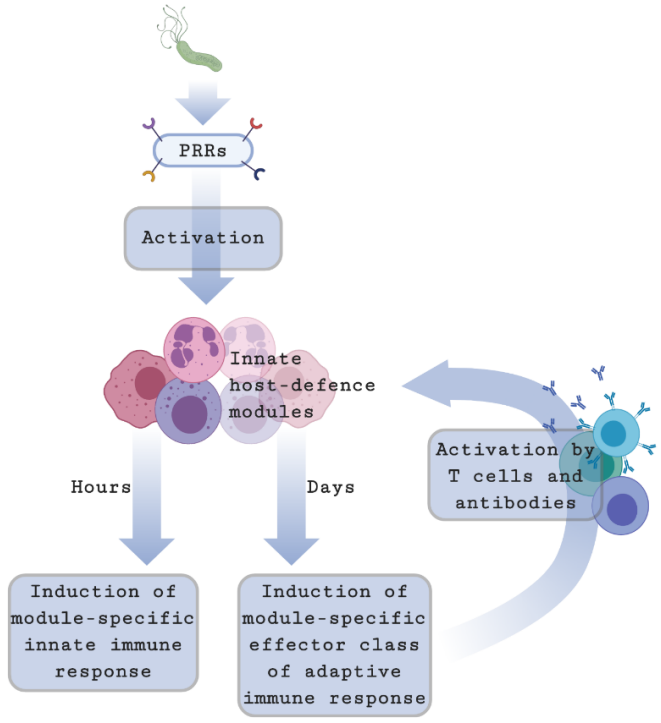
Figure 1.1| Overview innate and adaptive immune response in infected gastric tissue
[-A-] Innate immune response provides a first and fast layer of defense. Receptors specifically recognizing foreign molecules activate signaling cascades that lead to induction of proinflammatory cascades. [-B-] Among these bacterial factors, an LPS-derived metabolite was recently identified to activate the kinase ALPK1 and subsequently induce inflammation. [-C-] Lasting infection leads to recruitment of the adaptive immune system with B cells and T cells originating from lymphoid organs. Development and activation of these cells is tightly regulated. [-D-] The ligand PD-L1 is a known regulator, especially in the context of infectious disease. [-E-] The gastric pathogen *H.pylori* represents one of the most successful human immune invaders, infecting approximately 50% of the world's population and being able to persist for a lifetime, thereby increasing the risk for malignant clinical consequences.

Chapter

2. Background

2.1. [A] Innate immune system

During the first hours of exposure to a pathogenic organism, specific B cells and T cells are too few in number and need to be clonally selected and expanded to mount an effective immune response. Therefore, vertebrates rely on the phenotypically ancient and fast innate immune system. It is based on germline-encoded receptors, called pattern recognition receptors (PRR). They recognize structures that are uniquely associated to microbes, the pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) released from injured cells. PPR can be found on various cells which form distinctive modules, the effectors of the innate immune system (Figure 2.1). They carry out different functions such as elimination of foreign particles or injured cells, and the release of mediator substances to recruit other innate immune cells. Unlike the adaptive immune system, the innate immune system is not a single entity. It is a collection of distinct subsystems, or modules, that appeared at different stages of evolution and carry out different functions in host defense (reviewed in (Medzhitov 2007, Alberts 2014)).



Innate host-defense subsets can be activated directly by recognition of antigens with PRRs or indirectly by adaptive immune cells or compounds. Each subset can mount a fast and specific antimicrobial response. A module-specific adaptive immune response leads to activation of the same innate immune subset orchestrating the adaptive immunity. (Adapted from (Medzhitov 2007).)

Figure 2.1 | Activation of innate immune modules

2.1.1. Pathogen-associated molecular patterns

PRRs can be found transmembranous on a variety of cell types, leading to different effects. On phagocytic cells, such as macrophages and neutrophils, signaling can lead to uptake and subsequent destruction via phagocytosis. Intracellular PRR detect pathogens such as viruses and intracellular bacteria; and secreted PRRs mark pathogens for destruction by other cells.(Alberts 2014) In vertebrates, four major families have been reported so far – (**1**) Toll-like receptors (TLRs), (**2**) nucleotide-binding oligomerization domain (NOD)-Leucin Rich repeats-containing receptors (NLRs), (**3**) retinoic acid-inducible gene 1 (RIG-1)-like receptors (RLR), and (**4**) C-type lectin receptors (Walsh, McCarthy et al. 2013).

Toll like receptors

Toll-like receptors (TLRs) are evolutionary highly conserved and harbor an ectodomain with leucine-rich repeats that mediate recognition. They are expressed in immune and nonimmune cells, including fibroblasts and epithelial cells. Expression is modulated by response to pathogens, cytokines and environmental stresses. Cell surface TLRs (TLRs 1, 2, 4, 5, 6, and 10) recognize microbial membrane compartments, such as bacterial lipopolysaccharide (LPS) for TLR4 (Poltorak, He et al. 1998) and flagellin for TLR5 (Hayashi, Smith et al. 2001). TLRs, found in intracellular compartments (TLRs 3, 7, 8, and 9) recognize nucleic acids derived from bacteria or viruses and self-nucleic acids in disease conditions. Ligand recognition leads to adaptor protein recruitment such as MyD88 and TRIF which results in activation of pro-inflammatory transcription factors like NF-κB, IRFs and MAP kinases (Kawasaki and Kawai 2014).

NOD-like receptors

NOD-like receptors (NLRs) possess leucin rich repeats to mediate recognition. Primarily found in immune cells, they can also be expressed by nonimmune cells including epithelial and mesothelial cells. PAMP recognition via NLR leads to activation of NF-κB, MAPKs and caspase-1. Of the 22 known human NLRs, NOD1 and NOD2 are the best characterized and prototypical for NLR-mediated activation: bacterial peptidoglycan derivatives lead to self-oligomerization

to recruit and activate adaptor protein RICK, essential for activation of NF-κB and MAP kinases (reviewed in (Chen, Shaw et al. 2009)). Certain NLRs such as NLRP1 and NLRP3 form multiprotein complexes termed inflammasomes. The diverse set of signals leading to NLR-driven inflammasome formation include lethal toxin of *Bacillus anthracis* for NLRP1 and asbestos for NLRP3. Recognition leads to oligomerization and recruitment of the adaptor protein ASC which results in formation of the catalytically active caspase-1. This protease leads to maturation of the pro-inflammatory cytokines IL-1β and IL-18 and cleavage of gasdermin D, inducting pyroptosis, the lytic and inflammatory form of programmed cell death (reviewed in (Broz and Dixit 2016)).

Retinoic acid-inducible gene 1 -like receptors

Retinoic acid-inducible gene 1 (RIG-1)-like receptors (RLR) family, consisting of RIG-1, MDA5 and LGP2, signal in response to viral and bacterial nucleic acids (Newton and Dixit 2012). The cytosolic receptors are typically maintained at low level in a variety of cell types, including myeloid cells, epithelial cells and cells of the central nervous system. Upon exposure with cytokines and after virus infection, they are greatly increased to drive production of type 1 interferon (IFN1) cytokines (Loo and Gale 2011). RIG-I and MDA5 initiate the earliest response to viral infection: PAMPS such as dsRNA lead to RLR interaction with MAVS, the mitochondrial antiviral signaling proteins. MAVS forms a protein complex which catalyzes the kinases IKK and TBK1 resulting in phosphorylation, dimerization and subsequent nuclear translocation of the transcription factors interferon regulatory factor 3 and 7 (IRF3 and IRF7), activating the IFN1 signaling cascade (Brisse and Ly 2019).

C-type lectin receptors

C-type lectin receptors recognize carbohydrate structures in a mainly calcium-dependent manner with their C-type lectin-like domain. The trans-membranous receptors are largely found on immune cells but also nonimmune cells such as endothelial and epithelial cells. They are best characterized for their fungal recognition by binding to carbohydrates polymers such as mannan, glucans and chitins which are present in fungal cell walls (Goyal, Castrillón-Betancur et al.

2018). Others specifically bind damaged cells, oxidized lipids and self-alterations indicative of abnormality (Sancho and Reis e Sousa 2012).

2.1.2. Modules of the innate immune system

Unlike the adaptive immune response which consists of a specific response mediated by B cell sand T cells, the innate immune system is a collection of various subsystems which get rapidly activated in a stereotypical way through PRR activation (Figure 2.2). Different modules of the innate immune response have to act synergistically to build up an effective first line of defense.

Epithelial surface

The epithelial surface, region of first encounter with pathogens, provides already physical and chemical barriers: tight junctions between cells, antimicrobial molecules, mucus lining to avoid pathogenic adherence or commensal microbes that compete for nutrients with pathogenic intruders (reviewed in (Alberts 2014)). An important group of antimicrobial peptides are the mucosal derived defensins, which are continuously or inducibly expressed by microbial components or in inflammatory response (Ouellette 2004).

Cytokines

Important mediator substances are cytokines which can be produced by numerous cell types (Cohen, Bigazzi et al. 1974). They have a broad spectrum of biological functions beyond innate immune response such as recruitment of adaptive immune cells and driving hematopoiesis. Cytokines are major modulators of inflammation. They participate in acute and chronic inflammation via a complex interaction network. Given their diverse nature and function, many cytokines can be classified into several overlapping groups, such as illustrated in the following table.

Table 2-1 | Selection of cytokine classes with their corresponding effects and examples

Functional Class	**Primary Property**	**Other Effects**	**Examples**
Th1 cytokines	↑ Th1 response	clonal expansion of CTL	IFNγ, IL-2, IL-12, IL-18
Th2 cytokines	↑ Th2 responses	↑ antibody production	IL-4, IL-5, IL-18, IL-25, IL-33
Th17 cytokines	↑ Th17 responses, IFNγ	autoimmune responses	IL-17, IL-23, IFNγ
pro-inflammatory cytokines	↑ inflammatory mediators	↑ innate immune responses	IL-1α, IL-1β, IL-8, TNFα, IL-12, IL-18, IL-23
anti-inflammatory cytokines	↓ inflammatory genes	↓ cytokine-mediated lethality	IL-10, IL-13, TGFβ, IL-22, IL-1Ra, IFNα/β
gp130 signaling cytokines	growth factors	B-cell activation, acute phase	IL-6, CNTF, IL-11, LIF, CT-1
type II interferon	macrophage activation	increase class II MHC	IFNγ
type I interferons	anti-viral; ↑ class I MHC	anti-inflammatory, anti-angiogenic	IFNα, IFNβ

Adapted from (Dinarello 2007)

Phagocytes

To get rid of pathogens, cell debris or other harmful molecules, the phagocytic uptake and subsequent elimination is crucial for host defense. Phagocytes are macrophages which reside in most vertebrate tissues. Short-lived neutrophils, which reside in blood and get recruited to sites of infection where they contribute with pro-inflammatory cytokines. These phagocytes possess, in addition to their PRRs, a panel of receptors that lead, upon binding, to engulfment and subsequent phagocytosis (Medzhitov 2007).

Complement system

The complement system is a multiprotein complex of acute-phase proteins that include collectins, ficolins and pentraxins. They are markedly increased at early stages of infection and play an important role for the initiation and orchestration of phagocytosis. This system is composed of plasma proteins and operates in the bloodstream, tissues, or within cells to induce a series of inflammatory responses. Depending on the context, three distinct pathways can initiate it, leading to a

signaling cascade that includes (a.) attachment of C3 fragments resulting in opsonization of pathogens, marking them for phagocytosis (b.) release of fragments C4 and C5 which harbor a chemotactic activity leading to recruitment of phagocytes, and (c.) the formation of membrane-attack complex, resulting in the disruption of pathogens (Merle, Church et al. 2015).

Natural killer cells

Natural killer cells (NK) cells are leukocytes that are recruited early to sites of inflammation and induce apoptosis in abnormal host cells. They furthermore produce cytokines, especially IFNγ and harbor receptors controlling their activation, proliferation and effector function. They are closely related to T cells with a common progenitor and a similar killing mechanism as cytotoxic T cells by using perforins and granzymes. However, they lack the ability to generate antigen-specific receptors by genetic alterations (reviewed in (Lanier 2005)).

Dendritic cells

Dendritic cells *(DCs),* which are a heterogenous class of cells, serve as a major link to the adaptive immune system. They are distributed in tissues and organs and express a variety of PPRs. Upon PAMP recognition, they capture and process antigens. The activated dendritic cell migrates to nearby lymphoid tissue, like peyer`s patches or lymph nodes. Here it presents the antigen mounted on MHC class II molecules to passing organ where it activates T cells. If a T-cell receptor recognizes the presented antigen it gets activated. DCs undergo a stimulus-dependent maturation which can be triggered by foreign molecules but also adaptive immune cells. Besides the recruitment of T cells, they produce cytokines, such as IL-10 and the NK cell mobilizing cytokine IL-12 (reviewed in (Steinman and Hemmi 2006)).

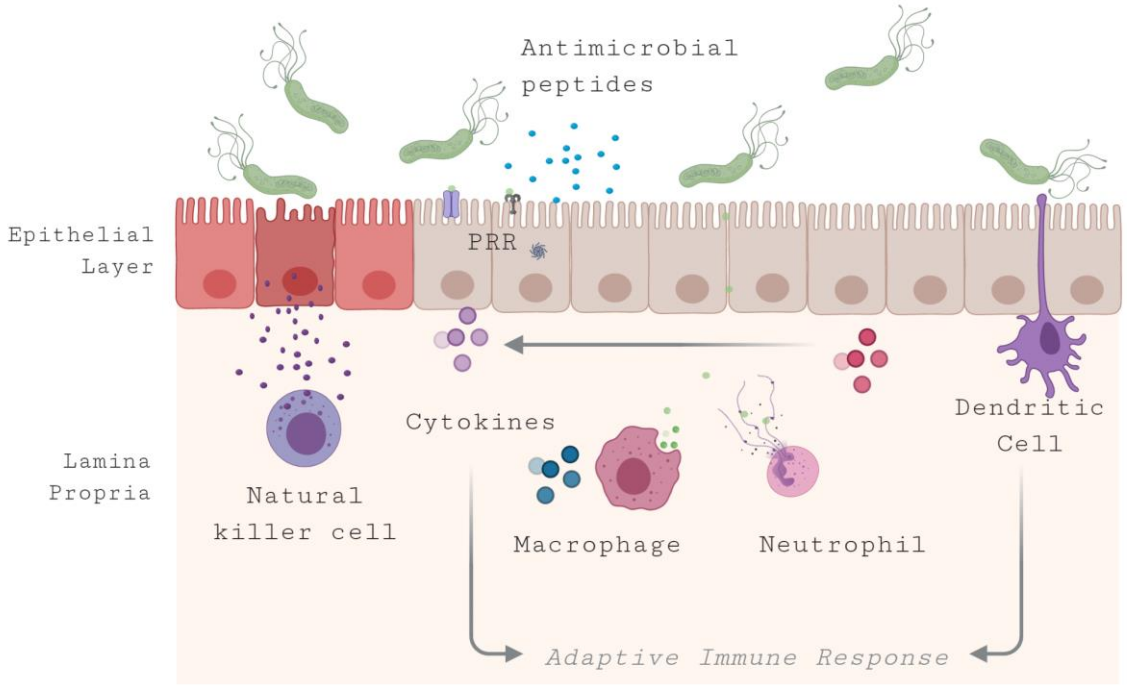


Figure 2.2 | Selection of innate immune response modules in bacterial infected tissue
A first layer of defense by epithelial cells is provided by antimicrobial peptides like defensines. Germ-line encoded PRRs such as TLRs and NLRs recognize bacterial components and trigger release of immune cell recruiting cytokines, such as IL-8. Neutrophils and macrophages act as phagocytes, eliminating bacterial particles which penetrate the epithelial lining. NK cells sense abnormal, infected cells leading to the release of apoptosis-inducing substances such as granzymes. DCs process and present antigens. They are able to recruit NK cells via release of IL-12 and lead, together with various cytokines to elicitation of adaptive immune responses.

2.2. [B] Innate immune regulator Alpha kinase 1

The transcription factor NF-κB is a central regulator of the immune response and plays a critical role in the host defense against pathogens. It can be activated by many danger-sensing receptors of the innate and adaptive immune system and activation mediates expression of a variety of genes involved in proliferation, survival, differentiation and proinflammatory cytokines. It thereby plays a critical role in the host defense against pathogens. Infection with the gastric bacterium *Helicobacter pylori* (*H. pylori*) is characterized by NF-κB activation and inflammation (Backert and Naumann 2010). Recently it was shown, that HBP, an intermediate metabolite of LPS synthesis, is transported via the bacterial secretion system and actives a signaling cascade, including the kinase ALPK1 to promote NF-κB activation (Zimmermann, Pfannkuch et al. 2017).

2.2.1. Immune regulator Nuclear factor-κB (NF-κB)

Identified as 'nuclear factor, binding near the κ light-chain gene in B cells', it was termed NF-κB (Sen and Baltimore 1986). Over thirty years of research later, it is now known to be a central element of any multicellular organism, important to adapt to chemical, mechanical and microbiological stress by regulating expression of around 600 genes (Gilmore). Thereby, it controls pathways involved in cell survival, differentiation and proliferation in the immune system, skeletal system and epithelium (Hayden and Ghosh 2012). Interestingly and highlighting its complexity, it participates in the regulation of seemingly contradictory pathways, such as both, inflammation onset as well as its resolution. Whereas onset is driven by the proinflammatory cytokines TNFα, IL-1β and IL-8, inflammatory resolution is mediated through anti-inflammatory prostaglandin PGD2 and the anti-inflammatory cytokine TGFβ1 (Lawrence, Gilroy et al. 2001).

Family members

NF-κB represents a family of ubiquitous inducible transcription factors which exist as homo- or heterodimers in the cytoplasm. The NF-κB signaling family consists of five structurally related proteins: (1) RelA or p65, (2) RelB, (3) c-Rel, (4) NF-κB1 or p50 and its precursor p105, and (5) NF-κB2 or p52 and its pre-cursor p100 (Ghosh, May et al. 1998, Hayden and Ghosh 2008). Through the commonly shared Rel homology domain, they dimerize to form at least 12 different identified forms and are able to bind to κB DNA elements, present in enhancer or promoter regions of target genes. Additionally, the three Rel proteins (RelA, RelB, c-Rel) harbor a C-terminal transactivation domain (TAD), which confers the ability to initiate transcription (Christian, Smith et al. 2016).

Signaling

In unstimulated condition, NF-κB exists in an inactive state, inhibited by ankyrin repeat-containing proteins. Based on the NF-κB dimer composition, two distinct pathways exist; a canonical and a noncanonical. Canonical signaling is induced through engagement of ligands with innate receptors, such as several cytokine receptors, and PRRs, but also with receptors of T cells (TCR), B cells (BCR) and of

costimulatory origin (Liu, Zhang et al. 2017). The typical pathway (Figure 2.3) consists of a dimer composed of p65 and p50, being sequestered in the cytoplasm by a member of the 'inhibitor of NF-κB' (IκB) protein family; IκBα, IκBβ or IκBγ. Appropriate ligand recognition leads to activation of the trimeric complex 'IκB kinase' (IKK), composed of three subunits: α, β, and a regulatory subunit γ ('NF-κB essential modulator', NEMO) (Karin and Ben-Neriah 2000). Phosphorylation of IκB by IKK leads to ubiquitination and subsequent degradation via proteasome (Ben-Neriah 2002). Degradation takes place within minutes, resulting in free NF-κB and translocation to the nucleus to regulate gene expression (Baeuerle and Baltimore 1988). As an autoregulatory pathway, this leads to upregulation of the inhibitor IκB, thus providing feedback mechanism to terminate NF-κB signaling (Sun, Ganchi et al. 1993).

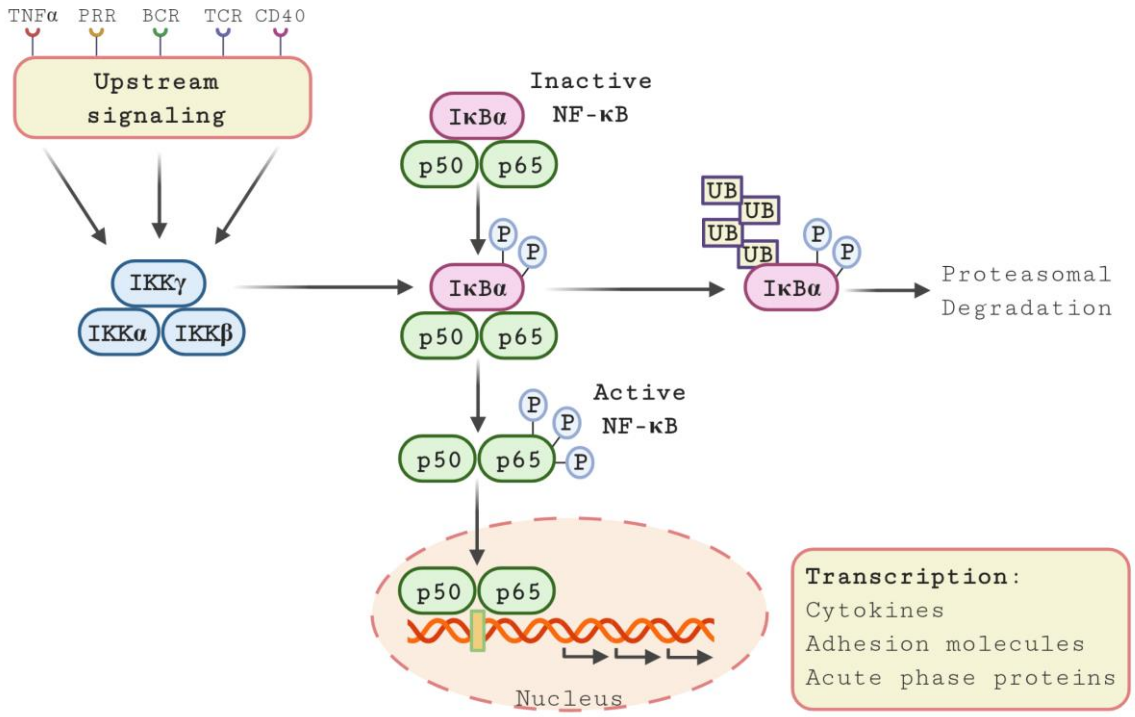


Figure 2.3 | Canonical NF- κB signaling pathway
Upon engagement of various receptors, the trimeric kinase complex IKK gets activated and phosphorylates IκBα which results in its ubiquitination and degradation. The released NF-κB gets phosphorylated, adjusting its transcriptional activity (reviewed in (Christian, Smith et al. 2016)) The complex is translocated into the nucleus and binds to κB elements of the DNA, regulating transcription of target genes. (Adapted from (Backert and Naumann 2010).)

In contrast to the canonical pathway being activated through degradation of IκB, the alternative pathway relies on proteasome-mediated processing of the NF-κB

family members p100 and p105 which contain an IκB-like C-terminal part, thus functioning as inhibitors (Sun 2011). Another difference is the relatively small number of receptors activating it with only some members of the TNF receptor superfamily, such as BAFFR, CD40, lymphotoxin β receptor and RANK (Christian, Smith et al. 2016). Both pathways seem to play distinct roles: at sites of inflammation the canonical NF-κB pathway activity is usually found, whereas the alternative NF-κB pathway was found to be important for B cell maturation and formation of secondary lymphoid organs (Senftleben, Cao et al. 2001).

2.2.2. NF-κB inducer heptose-1,7-bisphosphate (HBP)

Gaudet and coworkers identified the LPS-derived monosaccharide D-glycero-D-manno-heptose-1,7-bisphosphate (HBP) as a new inducer driving NF-κB activation in strains of the Gram-negative bacteria *Neisseria* (Gaudet, Sintsova et al. 2015). LPS' structure is highly conserved across different Gram-negative bacteria. Structurally, it can be divided into three domains: lipid A, core polysaccharide and O-antigen repeats, as illustrated in Figure 2.4 for *H. pylori* LPS (Alexander and Rietschel 2001). The hydrophobic lipid A domain anchors LPS to the bacterial outer membrane. Extended from lipid A, the hydrophilic surface layer composed of core polysaccharides and O-antigen repeats interacts with the surrounding environment. The conserved core domain can be subdivided into an outer core, made of hexoses and hexosamines and an inner region composed of 3-deoxy-D-*manno*-oct-2-ulsonic acid (Kdo) linked to *L,D*-heptose units.

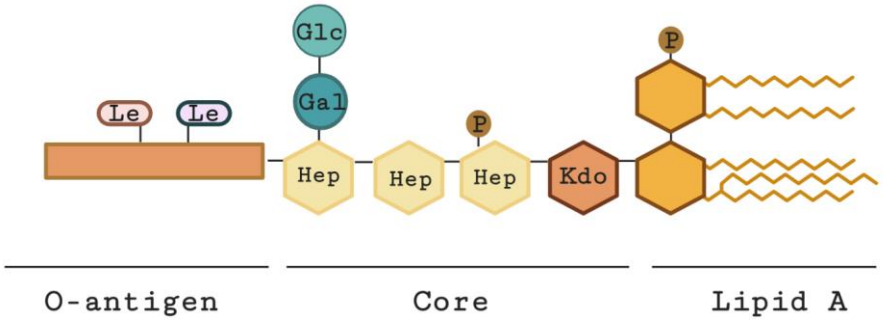


Figure 2.4 | Architecture of *H. pylori* derived LPS
LPS, derived from *H. pylori* consists of three compartments: Lipid A, which is linked to the outer bacterial membrane, a core polysaccharide composed of an inner and outer core and the O-antigen which often carries Lewis antigens (Altman, Chandan et al. 2011). Adapted from (Gall, Gaudet et al. 2017)

Highly conserved among Gram-negative bacteria yet absent in eukaryotes, the four- to five-step biosynthesis pathway of ADP heptose (Figure 2.5) is a necessity to transfer ADP-D,D-heptose or the epimer ADP-L,D-heptose to Kdo during *de novo* LPS synthesis. In the second step of this pathway, the intermediate metabolite HBP is synthesized via the kinase RfaE in *H. pylori* (Kneidinger, Marolda et al. 2002).

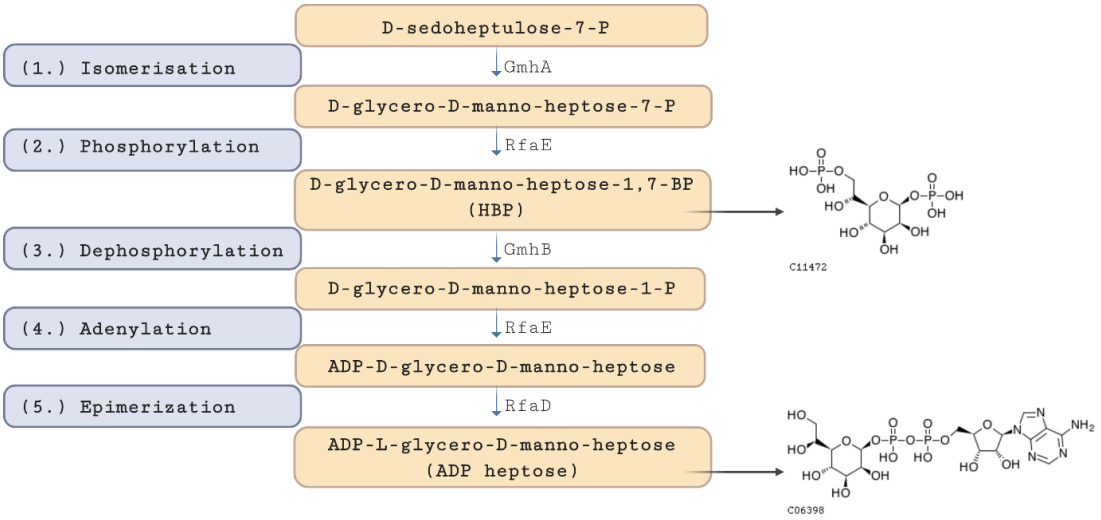


Figure 2.5 | ADP heptose synthesis pathway in *H. pylori*
(1.) D-sedoheptulose 7-phosphate is converted to D-glycero-α,β-D-manno-heptose 7-phosphate by the phosphoheptose isomerase GmhA, *(2.)* which gets phosphorylated at C1 by the bifunctional kinase RfaE. *(3.)* The resulting D-glycero- β -D-manno-heptose-1,7-bisphosphate (HBP) is converted to D-glycero-D-manno-heptose 1-phosphate by removal of the C-7 phosphoryl group through the phosphokinase GmhB. (4.) Adenylyl-transferase activity of RfaE leads to ATP transfer onto the C-1 position generating ADP-L-glycero-D-manno-heptose. (5) Activity of the epimerase RfaD results in the stereoisomer ADP-D-glycero-D-manno-heptose (ADP-Heptose). Adapted from (Chang, Wang et al. 2011). Molecule structures are taken from KEGG compound database.

2.2.3. HBP sensor alpha kinase 1 (ALPK1)

In 2017, two independent research groups showed with a range of different Gram-negative bacteria (*Shigella flexneri*, *Salmonella typhimurium*, *Neisseria meningitidis*, *H. pylori*) that HBP is recognized by protein alpha kinase 1 (ALPK1) of infected host cells (Milivojevic, Dangeard et al. 2017, Zimmermann, Pfannkuch et al. 2017). This enzyme belongs to the alpha kinase family, which was discovered through a database search for new kinases and was named after the mode of substrate specificity by its initial members, targeting amino acids located

within α helices (Ryazanov, Pavur et al. 1999). ALPK1, at first identified in lymphocytes and named accordingly, lymphocyte α-kinase, was later on found to be widely distributed in various cells (Ryazanov 2002). Residing on golgi-derived vesicles, it has been implicated in epithelial cell polarity and exocytic transport by phosphorylation of myosin IA, a motor protein regulating the delivery of vesicles towards the apical plasma-membrane (Heine, Cramm-Behrens et al. 2005). ALPK1 gene is a susceptibility locus for a range of diseases including gout (Wang, Tu et al. 2011, Ko, Tu et al. 2013), chronic kidney disease(Yamada, Nishida et al. 2013), type 2 diabetes mellitus (Shimokata, Oguri et al. 2013) and myocardial infarction (Fujimaki, Horibe et al. 2014). Development of these diseases share a common characteristic, chronic inflammation. First direct connections between ALPK1 and inflammation were provided in cell lines for Leydig cells (TM3), monocytes (THP1) and kidney cells (HEK293) harboring the knockdown which led to a decrease in mRNA transcripts of the pro-inflammatory cytokines IL-1β, TNFα and IL-8 (Wang, Tu et al. 2011, Kuo, Yeh et al. 2015). The only pro-inflammatory activator for ALPK1 is HBP, leading to a canonical NF-κB signaling cascade, as illustrated in Figure 2.6. For both, invasive bacteria such as *Shigella flexneri* or *Salmonella typhimurium*, as well as extracellular pathogens like *Neisseria meningitidis* or *H. pylori* the necessity of ALPK1 was shown for the activation of NF-κB (Milivojevic, Dangeard et al. 2017, Zimmermann, Pfannkuch et al. 2017).

NF-κB signaling cascade by HBP

HBP gets transported into the host cell, where it activates ALPK1 which directly or indirectly phosphorylates TIFA, the 'TRAF-interacting protein with forkhead-associated (FHA) domain-containing protein A' (Zimmermann, Pfannkuch et al. 2017). In TIFA overexpressing cells, it is observed that phosphorylated threonine is recognized by the FHA domain, leading to the formation of protein complexes, termed TIFAsomes, a necessity for downstream signaling (Huang, Weng et al. 2012). Consequently, in *Neisseria* infected cells, HBP was shown to be required for oligomerization (Gaudet, Sintsova et al. 2015). TIFA, which is present in the cytoplasm of most cells, was identified as a TRAF2- and later TRAF6-binding protein (Kanamori, Suzuki et al. 2002, Takatsuna, Kato et al. 2003). In line with this,

TRAF2 and the TRAF-activated protein TAB2 were found to be associated with TIFA protein complexes of host cells, infected with *H. pylori* (Zimmermann, Pfannkuch et al. 2017). In the Jurkat T cell line, treatment with HBP led to an activation of TIFA signaling through TRAF6 (Gaudet, Sintsova et al. 2015). 'Tumor necrosis factor receptor-associated factors', short TRAFs, are adaptor proteins, originally found to be recruited by receptors of the TNFR superfamily and Toll-like receptor-interleukin-1 receptor superfamily (Arch, Gedrich et al. 1998). Signaling by TRAFs leads to downstream activation of the 'transforming growth factor beta-associated kinase 1'(TAK1) complex, composed of TAK1 and TAK1-binding proteins (TAB1 and TAB2). Kinase activity of the TAK1 complex, leads to IKK phosphorylation (Wang, Deng et al. 2001). In line, *H. pylori* infection led to ALPK1-dependent IκBα phosphorylation. Thus, freeing NF-κB and leading to its nuclear translocation und upregulation of target genes, as illustrated in Figure 2.6 (Zimmermann, Pfannkuch et al. 2017).

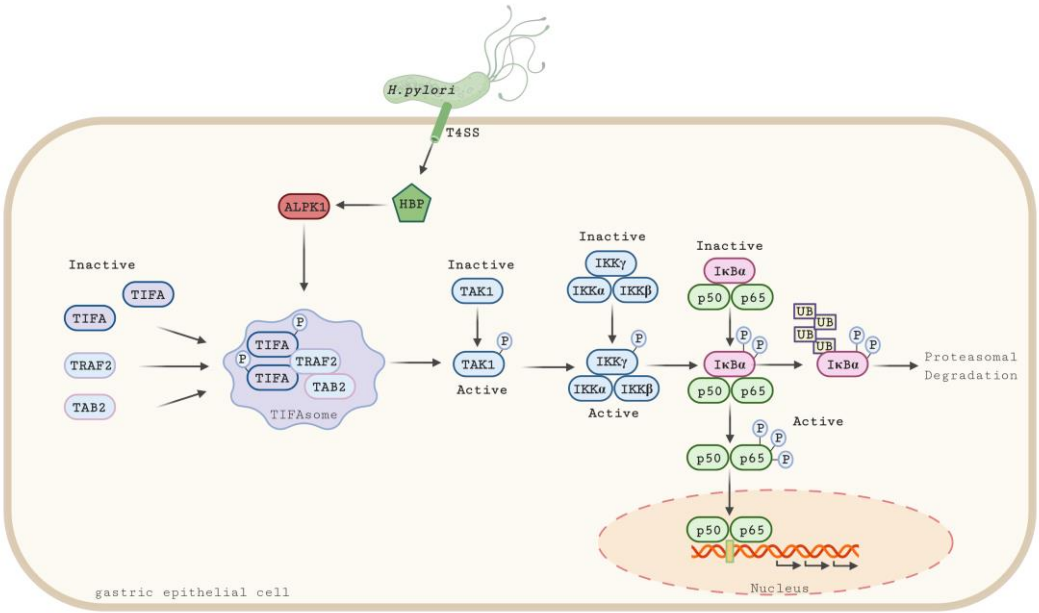


Figure 2.6 | HBP mediated induction of NF-κB in *H. pylori* infection
Infection with the Gram negative bacteria *H. pylori* and subsequent formation of the type 4 secretion system leads to transport of the LPS synthesis intermediate HBP into the host cell. There, HBP is recognized by the kinase ALPK1 which starts to trigger a signaling cascade. The protein TIFA gets phosphorylated and oligomerizes to a protein complex, called TIFAsomes containing TRAF2 and TAB2. Subsequent TAK1 activation results in IKK phosphorylation which frees the NF-κB complex by phosphorylation of the inhibitor IκBα. The subunits p65 and p50 translocate into the nucleus where they bind κB DNA region, resulting in induction of proinflammatory cytokines, such as IL-8 and TNFα. Adapted from (Zimmermann, Pfannkuch et al. 2017).

2.3. [C] Adaptive immune system

The second and more sophisticated line of defense, the adaptive immune system is based on the almost limitless variety of B and T cells. With this, essentially any molecule can be recognized in a highly specific manner. DNA rearrangement which leads to the high variety of lymphocytes, occurs randomly and produces thereby also receptors that bind host molecules. To avoid host cell damage, a process called immunological self-tolerance has evolved.

The adaptive immune response can be divided into two classes; the B cell mediated antibody response and the T cell-mediated immune response.

Multipotent hematopoietic stem cells, which are found in the bone marrow, give rise to common lymphoid progenitor (CLP) cells which can differentiate in T cells, B cells, NK cells and dendritic cells by a tightly regulated process (Kondo, Weissman et al. 1997). T cells develop in the thymus and B cells in the bone marrow. These sites of maturation are referred to as central (primary) lymphoid organs. Matured lymphocytes migrate to peripheral (secondary) lymphoid organs via the blood. There, in lymph nodes, spleen, epithelium-associated lymphoid tissue in the skin, respiratory tract and gastrointestinal tract, they can be activated by antigens.(reviewed in (Alberts 2014))

2.3.1. Antibody mediated immune response

In immune response, the two main functions of B cells is antibody production (humoral immunity) and to form the immunological memory. They are activated upon recognition of a specific antigen by their B-cell receptor (BCR), a complex of membrane-bound immunoglobulins (mIg).

Immunoglobulins

Immunoglobulins (Igs) without membrane binding domain are also referred to as antibodies and act as central mediators of immunity by directly neutralizing pathogens, their derived products and recruitment of immune effectors to eliminated infections and aberrant cells (Li, Yin et al. 2019). They are Y-shaped proteins containing an N-terminal antigen-binding surface and a C-terminal tail that gives further properties. The two identical antigen-binding regions consists of four

polypeptide chains, two light chains and two heavy chains. Both contain a constant region in their C-terminal amino acid sequence and a variable region in the antigen-binding N-terminal end. These are encoded by random DNA sequence assembly of gene segments that are called V, J for the light chain and additionally D for the heavy chain region, a process called V(D)J recombination (reviewed in (Alberts 2014)). In mammals, five major Ig classes exist; IgM, IgD, IgG, IgA, IgE, each with its own heavy chain, tissue distribution and biological properties. Secreted as pentameres or hexameres, IgM proteins mediate complement activation and have a strong affinity towards PAMPs. Due to their size, they are not able to pass into extravascular space. Monomeric Igs, such as IgG, IgE and IgA, which can also occur as dimer, are able to pass and be distributed to tissues (Li, Yin et al. 2019). The primary Ig repertoire consists of 10^{12} different IgM and IgD and is produced in the absence of antigen stimulation to ensure antigen recognition. Antigen stimulation leads to production of other classes of Igs, the secondary Ig repertoire. This class switching process and somatic hypermutation increases antigen binding affinity over time, and is called affinity maturation (Figure 2.7). The first Igs of developed B cell are incorporated into its plasma membrane as receptors, the B cell receptors (BCRs). A single BCR is composed of 1.5 x 10^5 Igs recognizing the same epitope (Maddaly, Pai et al. 2010).

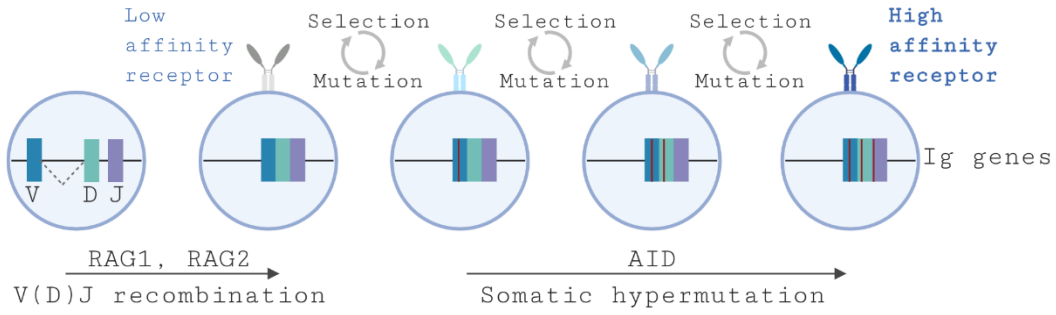


Figure 2.7 | V(D)J recombination and hypermutation of Ig genes
Mediators of V(D)J recombination are RAG1 and RAG2 enzymes. AID drives somatic hypermutation and class-switch recombination of Ig genes. Each process is characterized by several rounds of selection and mutation, yielding in high affinity immunoglobulins. (Adapted from (Greaves and Müschen 2015).)

Development

Development of B cells occurs in the fetal liver and bone marrow of adults. There, direct contact with bone marrow stroma is required for the B cell precursor pro-B cell to arise from common lymphoid progenitors (Maddaly, Pai et al. 2010). Several growth-promoting signals drive subsequent maturation, gene rearrangement rendering the cell to the pre-B cell stage with is characterized by the expression of a cell surface pre-B cell receptor (Melchers 2005). They undergo further DNA rearrangement, which involves the recombinase-activating genes (RAG) 1 and 2 to become immature B cells characterized by surface expression of IgM. The subsequent expression of IgD is a characteristic of naïve mature B cells. Mature B cells depart the bone marrow and recirculate among peripheral lymphoid tissues. There, they are able to give rise to memory B cells and antibody secreting plasma cells upon activation (Lou, Casali et al. 2015).

Activation

Naïve B cells in the periphery express both, BCRs for antigens as well as PRR to respond to PAMPs, such as the DNA-recognition receptor TLR9. Depending on the presence of PAMPs, there are two possible outcomes:

(1) In the presence of antigens and PAMPs, B cells are stimulated to proliferate into short-lived plasma cells. Internalization of antigens is blocked by PRR engagement (reviewed in (Akkaya, Kwak et al. 2020)). In terms of antibody production and memory induction, this response is considered rather weak, due to the lack of affinity maturation (Maddaly, Pai et al. 2010).

(2) In the presence of only antigens, the get internalized, processed and presented on MHC class II. During this process, they start to produce T cell attractants, such as a dihydroxylated forms of cholesterol (Cyster, Dang et al. 2014). Engagement with antigen-specific T cells leads to B cell proliferation and differentiation into short-lived plasma cells or formation of transient structures called germinal center B cells (MacLennan 1994). These tightly confined clusters of cells are the centers of B cell affinity maturation by class switch DNA recombination to change Ig class and somatic hypermutation to inserts point-mutation into Ig V region to further increase antigen binding affinity (Xu, Zan et al. 2012). This activation can result in

three fates: First, the differentiation towards long-lived plasma cells that reside in the bone marrow and secrete large amounts of antibodies. Without antigens or a second antigen challenge, they can persist for years or potentially a lifetime. Another possibility is the differentiation into long-lived memory B cells. These quiescent cells are found within secondary lymphoid organs and other tissues. B cells leaving the germinal center can thirdly re-enter to further increase antigen affinity (Akkaya, Kwak et al. 2020).

The peril of H. pylori infection

However, despite complex response to immune challenges, not all infection can be controlled or cleared. In context of the gastric pathogen *H. pylori*, infected patients show elevated level of bacteria-specific IgA and IgG antibody levels which are used as infection marker but they fail to control the infection (Ermak, Giannasca et al. 1998, Futagami, Takahashi et al. 1998). Moreover infection with several pathogens, such as *H. pylori* and *Plasmodium falciparum* was shown to drive formation of B cell lymphoma (Greaves and Müschen 2015). *H. pylori*-infection can lead to B cell lymphomas in mucosa-associated lymphoid tissue (MALT). Chronic colonization leads to constant exposure to antigens, resulting in potentially permanent germinal center formation including the affinity maturation which occurs in there. This increases the possibility of mutation to occur in non-Ig genes of B cells, as illustrated in Figure 2.8. (Marcelis, Tousseyn et al. 2019).

Figure 2.8 | Deletion and mutation of Non-Ig genes by *H. pylori*
H. pylori infection is characterized by induction of NF-κB, which is a key driver of normal B cell function, including transcriptional regulation of proliferation and maturation driving genes. Repetitive or chronic activation can lead to targeting of RAG1, RAG2, AID towards non Ig genes which can drive clonal evolution towards lymphoma (Marcelis, Tousseyn et al. 2019). (Adapted from (Greaves and Müschen 2015).)

2.3.2. T-cell mediated immune response

The second branch of adaptive immunity is mediated by T lymphocytes which facilitate a broad spectrum of tasks. One of the earliest discovered functions was providing help to antigen production of B cells, coining the term 'T helper cell' for a specific T cell subset (Claman, Chaperon et al. 1966). Along with adaptive immunity, many modules of the innate immunity branch are promoted as illustrated in Figure 2.9. They control infection with intracellular pathogens and tumorigenesis by promoting cell lysis and play a pivotal role in establishment of peripheral tolerance. To recognize antigens, T cells harbor either αβ- or γδ- T cell receptors (TCRs) which are distinct in antigen recognition and effector function (Chien, Meyer et al. 2014). In contrast to B cell receptors, which are able to recognize antigens in their native form, most TCRs only recognize processed antigens in the form of short peptides, expressed on the surface of antigen-presenting cells and bound to a major histocompatibility complex (MHC) (Alcover, Alarcón et al. 2018). Therefore, they evolved a sophisticated process for generating a huge repertoire of antigen receptors.

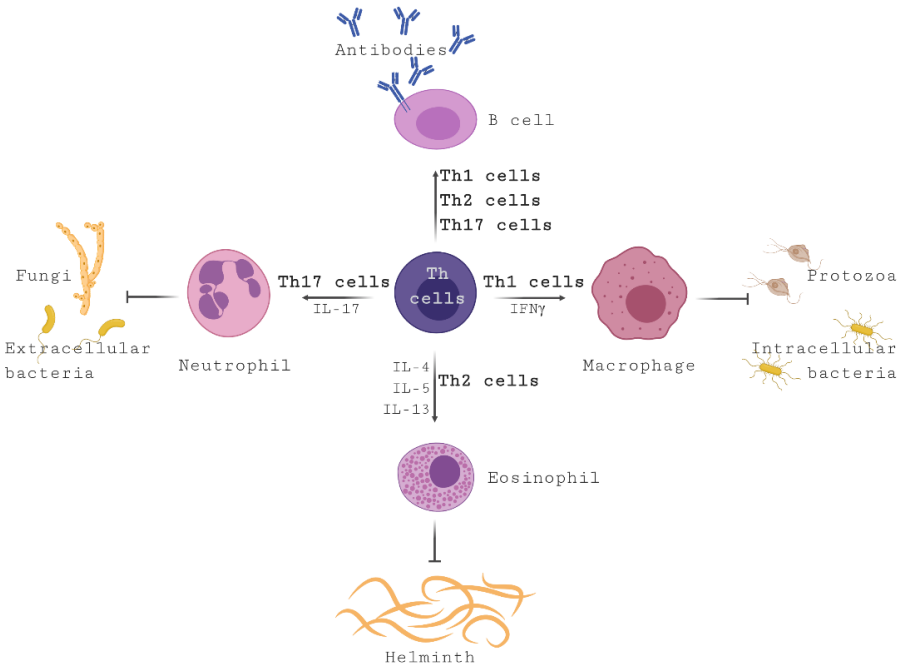


Figure 2.9 | Effector Th cells and promoted immune response
Upon antigen recognition, $CD4^+$ cells are able to differentiate into a distinct subpopulation of cells. These types differ in effector function. They are able to promote antibody production in B cells and recruit various modules of the innate immune system branch, required for different immune challenges, such as fungal or microbial infection. (Adapted from (Medzhitov 2007).)

Development

Origin of thymocytes are CLPs in the bone marrow, but thymopoiesis occurs in the thymus, where the progenitors migrate in waves, in early stages of embryogenesis (Haddad, Guimiot et al. 2006). There, cells start stepwise development after receiving signals from the thymus, stromal cells, epithelial cells, macrophages or dendritic cells (Anderson, Moore et al. 1996). Thymopoiesis steps are typically defined based on the surface expression of CD4 and CD8, starting with $CD4^-CD8^-$ as double negative when most cells start to rearrange their T cell receptor (TCR) gene locus and resulting in the assembly of a pre-TCR complex. In the following step they become $CD4^+CD8^+$and undergo a second round of gene rearrangement at their TCR. The resulting *double-positive thymocytes* migrate inward, where they either bind self-MHC peptides expressed by stromal cells with their TCR with a certain affinity or they undergo death by neglect. Negative selection eliminates T cells which could potentially attack healthy host cells and positive selection occurs to guarantee that the TCR are able to bind MHC molecules. This leads to *single-positive* $CD4^+$ or $CD8^+$*thymocytes* which exit the thymus as naïve T cells into the pool of T cells (Robey and Fowlkes 1994, Witt and Robey 2005).

Activation

To induce immunity, naïve T cells need to be activated by antigen-presenting cells (APCs) which display antigens after appropriate processing. $CD4^+$ T cells recognize protein-derived peptides in association with MHC class II on professional APCs such as DCs, macrophages, and B cells. $CD8^+$ T cells recognize peptides bound to MHC class I which are present of most nucleated cell (Blum, Wearsch et al. 2013). Another, minor class of T cells recognizes lipids, bound to the CD1 family (Cohen, Garg et al. 2009). Three signals are required that determine T cell fate upon activation. Signal 1 is mediated through the TCR upon binding to the appropriate peptide-MHC complex. Signal 2, referred to as “costimulation”, which is an accessory signal. Together with signal 1, it induces immunity, often measured in clonal expansion, differentiation into effector cells and long-term increase in precursors (‘memory T cells’). Absence of it results in anergic T cells which can

contribute to tolerance. Signal 3 determines the differentiation into an effector cell (Reis e Sousa 2006). Signal 2, which provides co-stimulation or -inhibition is transmitted via cell surface molecules that transduce T cells to positively or negatively regulate TCR signaling regions. The repertoire of co-signaling receptors on T cells is highly versatile and adaptive to environmental changes. Co-signaling ligands have been identified on nearly all types of cells. The most well-characterized families are the T cell receptor family CD28 which interact primarily with the B7 ligand family (Chen and Flies 2013).

Effector classes

Subsets of T cells exist upon activation. $CD8^+$ thymocytes are known as *cytotoxic T cells (Tc cells*). They play pivotal roles in the control of intracellular pathogens by promoting the apoptotic death of target cells. A mechanism that involves granules, such as perforin and granzyme, combined with death-receptors, such as Fas or tumor necrosis factor is needed (Maher and Davies 2004). $CD4^+$ T cells on the other hand, display a more diverse subset after activation: Th (T helper)1, Th2, Th9, Th17, Th22, Treg (regulatory T cells), and Tfh (follicular helper T cells). All are defined by different cytokine profiles in induction and effector function (Figure 2.10).

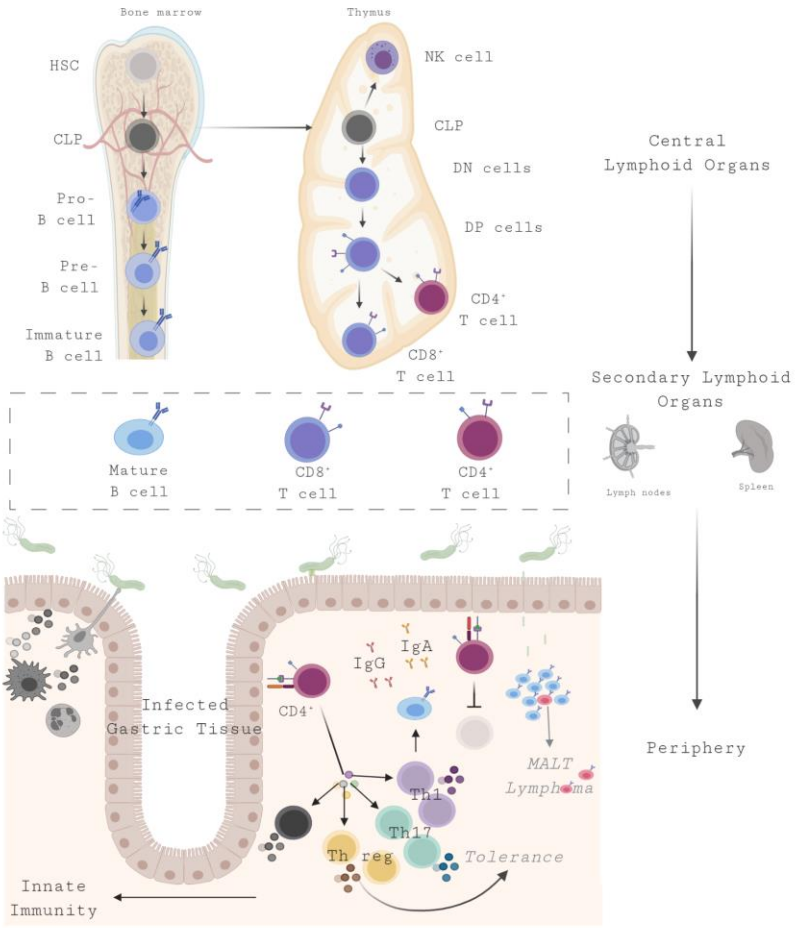


Figure 2.10 | Adaptive immunity in *H. pylori* infected tissue
Adaptive immune cells are produced in central lymphoid organs in tightly regulated processes. Native B cells a T cells migrate into secondary lymphoid organs and are subsequently distributes within the periphery. Upon infection, they are recruited by innate immune cells. B cell response is marked by IgM and IgA production and can lead to MALT lymphomas. T cell response consists mainly of Th1, Th17 and Th reg subsets. The latter is known to establish tolerance, promoting chronic infection by limiting immune response. (Adapted from (Reyes and Peniche 2019))

2.3.3. Self-tolerance

Tolerance is the ability of the immune system to respond to foreign antigens without attacking host molecules to protect against autoimmune tissue injury. To distinguish foreign molecules from self-molecules, a number of mechanisms exists in the adaptive immune system: (1) *receptor editing* changes developing B cells that recognize self-molecules, (2) *clonal deletion* leads to apoptosis of potentially self-reactive B and T cells once they encounter their self-molecule, (3) *clonal inactivation/clonal anery* leads to functional inactivation of reactive B and T cells and (4) *clonal suppression* is the regulatory T cell driven suppression of other potentially self-reactive lymphocytes.

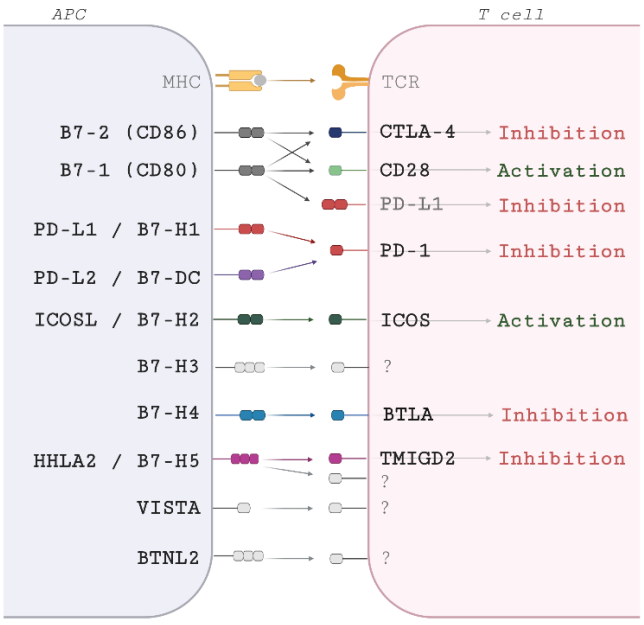
Processes operating in central lymphoid organs where the maturation of B and T cells takes place, as receptor editing and clonal deletion lead to ***central tolerance***. However, central tolerance alone is insufficient to prevent from autoimmune injuries as some cells still harbor autoreactive receptors. Tolerance establishing mechanisms in extrathymic, peripheral organs, such as clonal inactivation and suppression affect mature lymphocytes and lead to ***peripheral tolerance***. (Alberts 2014) (Rich 2019) Primary mediators of peripheral tolerance are Tregs which are characterized by the transcription factor FOXP3, required for development, maintenance and function. They are crucial for preventing autoimmune disease like type 1 diabetes and controlling chronic inflammation as occurring in asthma or inflammatory bowel disease. However, presence of Tregs can also prevent beneficial responses in limiting immune response to certain pathogens or tumorigenic cells (Vignali, Collison et al. 2008). Another mechanism to ensure discrimination of self from non-self is the 'two-signal' concept of lymphocyte activation (Bretscher and Cohn 1970). Engagement of T cell or most B cell receptors alone is not sufficient to promote activation. Additional signaling provided by co-stimulatory receptors is required. The most well-characterized superfamily is B7:CD28.

2.4. [D] Adaptive immune regulator Programmed death-ligand 1

As a member of the B7:CD28 superfamily of accessory molecules, Programmed death-ligand 1 / PD-L1 serves as signal 2 in Bretscher and Cohn's 'two-signal' model, which is needed by most lymphocytes and thereby critical for a functioning immunity. Specifically, it is important for lymphocyte development, control of excessive or damaging inflammation and to ensure tolerance against self-molecules. However, aberrant expression is found in a range of disease, such as autoimmune disease and cancers, rendering the immune response ineffective in the affected tissue. In the past decade, targeting PD-L1 and other accessory molecules -an approach termed immune checkpoint inhibition- has revolutionized the field of cancer treatment with promising results, especially for patients with advanced tumor stages with traditionally poor prognosis.

B7:CD28 – A superfamily of regulators

The B7 family of ligands interacts with the CD28 receptor family (Figure 2.11), forming the B7:CD28 superfamily, determining lymphocyte activity towards activation or inhibition. The two first describes and best characterized receptors are CD28 (Aruffo and Seed 1987, Gross, St John et al. 1990) and CTLA-4 (Brunet, Denizot et al. 1987, Linsley, Brady et al. 1991). They exhibit opposing effects, with CD28 as activating and CTLA-4 as inhibitory accessory signal (Krummel and Allison 1995, Leach, Krummel et al. 1996). Ligands are the B7 family members B7-1 and B7-2, which are capable of binding to both receptors, thus balancing signaling. Differences in expression level, cell-type dependent occurrence and inducibility ensure a balanced regulation. Under unstimulated conditions, affinity for the inhibiting CTLA-4 receptor is higher, avoiding self-destructive immune responses (Linsley, Greene et al. 1994). Ligand expression levels differ in a cell-dependent manner, such as on APCs where B7-2 is constitutively expressed and upregulated rapidly serving as co-stimulator for initial immune responses, whereas B7-1 is found to be inducibly expressed later (Freeman, Gribben et al. 1993, Hathcock, Laszlo et al. 1994).



Receptors, primarily mediating a positive immune regulation are CD28 and ICOS, receptors for negative immunomodulation are CTLA-4, PD-1, TMIGD2 and BTLA. Although there are structural similarities among the receptors, functional heterogeneity is observed. The interaction network is complex: Some B7 ligands are able to bind to multiple receptors or even act themselves as receptor, binding to other B7 family members. Expression and distribution of B7:CD28 is highly divers and the number of family members grew over the last years, but many regulatory mechanism remain elusive. (Adapted from (Sharpe and Freeman 2002).)

Figure 2.11 | Interaction pathways in the B7:CD28 superfamily

Another feature of this superfamily is 'reverse signaling', a process when a ligand becomes a receptor. It has been described for the ligands B7-1 and PD-L1, whose interaction results in inhibited T cell proliferation (Butte, Keir et al. 2007). Over the past years, the number of members for the B7:CD28 superfamily has grown and one of the more recently delineated pathways is the PD-L1:PD-1 axis.

2.4.1. Structure & Expression

In cell lines of T cells and lymphoid/myeloid progenitor cells, undergoing programmed cell death, an immunoglobulin gene was identified as pivotal regulator for initiation of the process and was termed programmed cell death 1/PD-1. However, transfection of PD-1 gene failed to induce apoptosis, indicating towards a more complex mechanism (Ishida, Agata et al. 1992). Seven years later, a negative regulator for immune responses that increases production of the anti-inflammatory cytokine IL-10 while leading to increased T cells apoptosis was described and named B7 homolog 1/B7-H1 for its structural similarity with B7-1 and B7-2 (Dong, Zhu et al. 1999). B7-H1 became 'Programmed death-ligand 1'/PD-L1, when its binding to PD-1 was discovered (Freeman, Long et al. 2000). Later on, a second PD-1 ligand was discovered, named PD-L2 or B7-DC for its expression on dendritic cells (Latchman, Wood et al. 2001, Tseng, Otsuji et al. 2001).

PD-1 – the unusual CD28 receptor

Unlike CD28 and CTLA-4 whose expression is predominantly restricted to T cells, PD-1 expression is inducibly also found on B cells, NK cells, myeloid cells and DC subsets (Agata, Kawasaki et al. 1996, Finger, Pu et al. 1997, Liu, Yu et al. 2009, Pesce, Greppi et al. 2017).

As all receptors of the family share sequence similarities with CD28, PD-1 shares ~20 %. It is a type I transmembrane glycoprotein composed of an Ig Variable-type (V-type) extracellular domain, a trans-membranous domain, and a cytoplasmic tail, able to bind signaling and scaffolding molecules. It harbors four potential sites for N-linked glycosylation and its cytoplasmic tail contains two tyrosine residues in two highly functionally highly conserved sequence motifs; an N-terminal ITIM and a C-

terminal ITSM (Shinohara, Taniwaki et al. 1994, Finger, Pu et al. 1997, Vibhakar, Juan et al. 1997). Among all B7 family members, the PD-1 ligands display the widest tissue distribution with PD-L1 showing the broadest distribution and expression capacity (Yamazaki, Akiba et al. 2002, Brown, Dorfman et al. 2003).

PD-L1 – the omnipresent ligand

PD-L1, encoded by the gene CD274, is a 290 aa type I transmembrane protein. The IgV-like and the IgC-like domain are extracellular with the former being responsible for PD-1 interaction (Keir, Butte et al. 2008). No conserved motif is found in the intracellular tail but a study demonstrated the dependency to transmit antiapoptotic signals within cancer cells (Azuma, Yao et al. 2008).

Under physiological conditions, PD-L1 is constitutively expressed on a range of immune cells, including B cells, naïve T cells, DCs and macrophages. An exceptional role is given to PD-L1 by the ability to be inducibly expressed by various stimuli, as illustrated in Figure 2.12. As negative regulation of T cell activity is one of the major tasks of the PD-L1:PD-1 axis, T cell activity itself, drives PD-L1 upregulation. In naïve T cells, expression is markedly enhanced after anti-CD3 mAb stimulation (Yamazaki, Akiba et al. 2002) and especially Th1 response leads to upregulation of PD-L1 (Loke and Allison 2003). IFNγ, which is released by Th1 is considered to be the most potent soluble inducer as the transcription factor IRF1 acts directly at the promoter (Lee, Jang et al. 2006, Moon, Kong et al. 2017). However, ability to drive transcription is more complex. As it was shown for a sarcoma mouse model, where treatment with IFNγ-blocking antibodies significantly reduced PD-L1 expression on tumor cells but only marginally on tumor-associated macrophages (Noguchi, Ward et al. 2017). Likewise in human melanoma cells, where IFNγ-induced PD-L1 expression was shown to be NF-κB dependent (Gowrishankar, Gunatilake et al. 2015). Apart from immune cells, inducible expression is shown in cells of non-lymphoid origin, including endothelial cells(Mazanet and Hughes 2002) or epithelial cells (Nakazawa, Dotan et al. 2004, Schoop, Wahl et al. 2004). In the last years, many other PD-L1 expression drivers were identified, such as bacterial-derived LPS (Yamazaki, Akiba et al. 2002),

oncogenes such as c-myc (Casey, Tong et al. 2016) or hypoxia (Noman, Desantis et al. 2014).

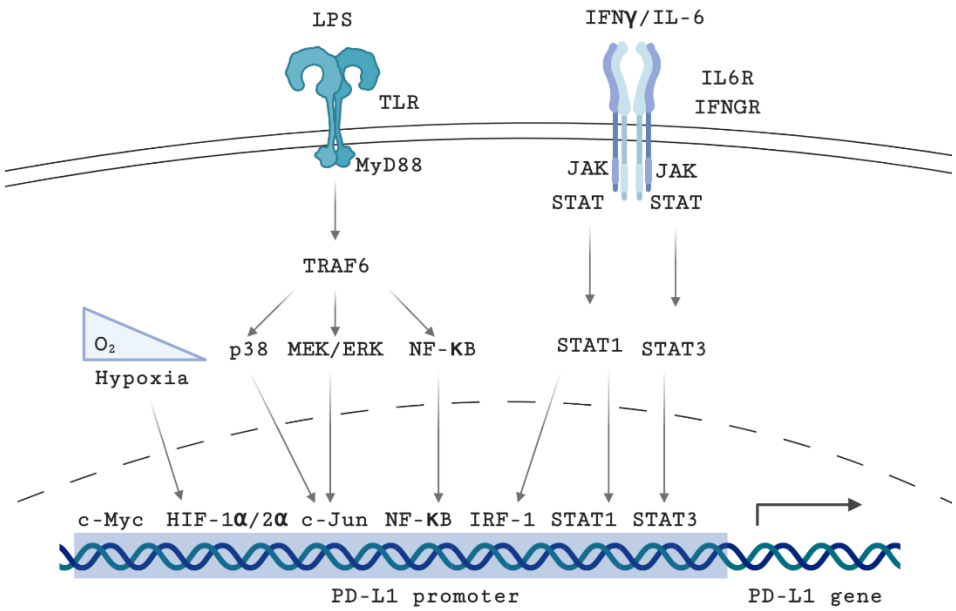


Figure 2.12 | Transcriptional regulation of PD-L1
The promoter of PD-L1 has various binding sites, giving the ability to be rapidly induced upon stimulation. Stimuli, such as the bacterial compound LPS or the cytokines IFNγ and IL-6 were shown to trigger signaling cascades, which leads to shuttling of transcription factors, such as c-Jun, NF-κB, IRF-1, STAT1, STAT3 into the nucleus, directly binding to the promoter, thereby enhancing PD-L1 transcription. Additional factors, such as hypoxia or c-myc are similarly able to drive gene expression. (Adapted from (Zerdes, Matikas et al. 2018).)

2.4.2. Signaling axis of PD-L1 : PD-1

ITRMs –orchestrators of immune receptor signaling

Immune cells communicate with the environment through their immunoreceptors, which are often characterized by intracellular tyrosine residues, that get phosphorylated upon receptor activation and serve as docking regions to recruit downstream signaling proteins (Guy, Vignali et al. 2013). These immunoreceptor tyrosine-based regulatory motifs (ITRMs) include inhibitory (ITIMs), activating (ITAMs) and switching (ITSMs) motifs which are able to convey either activating or inhibiting signals. Activating and inhibitory motifs are often but not mandatorily found within one receptor sequence, as regulation can be intertwined. All motifs

were found to be phosphorylated by Src family kinases (SFKs) upon receptor ligation (Sidorenko and Clark 2003). Phospho-ITAM residues recruit one of the two SYK family members in a cell-dependent manner, with SYK for B cells and ZAP70 for T cells, initiating downstream events (Parsons and Parsons 2004, Bezbradica and Medzhitov 2012). Phosphorylation of the ITAM-antagonizing ITIM is usually initiated by Lyn with Phospho-ITIMs serving as docking station specific for phosphatases with SH2 domain (SHP-1, SHP-2 or SHIP), resulting in dephosphorylating of upstream molecules, including the receptor itself and recruited SFK and SYK (Ravetch and Lanier 2000). Downstream signaling differs among the phosphatases and affinity varies among motifs. Like ITIMs, phosphorylated ITSM (originally termed SLAM) binds SH2 domain-containing tyrosine phosphatases but they are capable to directly bind adaptor molecules, called SAPs (Sidorenko and Clark 2003). In general, binding of members of the 'signaling lymphocytic activation molecule (SLAM)-associates protein' (SAP) adaptor family, such as SH2 domain-containing protein 1A (SH2D1A) leads to SHIP recruitment, driving activating mechanisms, whereas its absence results in inhibitory signaling due to binding of other proteins, such as SHP-2 (Dong, Strome et al. 2002, Dong and Veillette 2010).

PD-1 signaling mechanism

Like in all CD28 members, activity of PD-1 is mediated by its cytoplasmic tail which serves as docking station for signaling proteins mediating downstream effects. The PD-L1:PD-1 cascade in B cells or T cells, as illustrated in Figure 2.13, requires two signals; (1) ligation of the BCR/TCR and (2) PD-L1:PD-1 engagement and can be divided into three initiating steps, culminating in the recruitment of phosphatase SHP-2 (Latchman, Wood et al. 2001, Okazaki, Maeda et al. 2001, Chemnitz, Parry et al. 2004). Another study could show, that not only SHP-2, but also SHP-1 were found associated with PD-1 (Chemnitz, Parry et al. 2004). Contradictory results were provided, as mice deficient for SHP-2 did not develop immunopathology upon viral challenging, hinting towards SHP-2-independent PD-1 signaling mechanism (Rota, Niogret et al. 2018). As both, ITIM and ITSM are able to bind SH2 domain-containing tyrosine phosphatases, sequence mutation were

performed to reveal signaling responsibility. ITSM was identified as the motif responsible for inhibition of B cells or primary human CD4 T cells, since ITIM mutations had negligible effects (Okazaki, Maeda et al. 2001, Chemnitz, Parry et al. 2004).

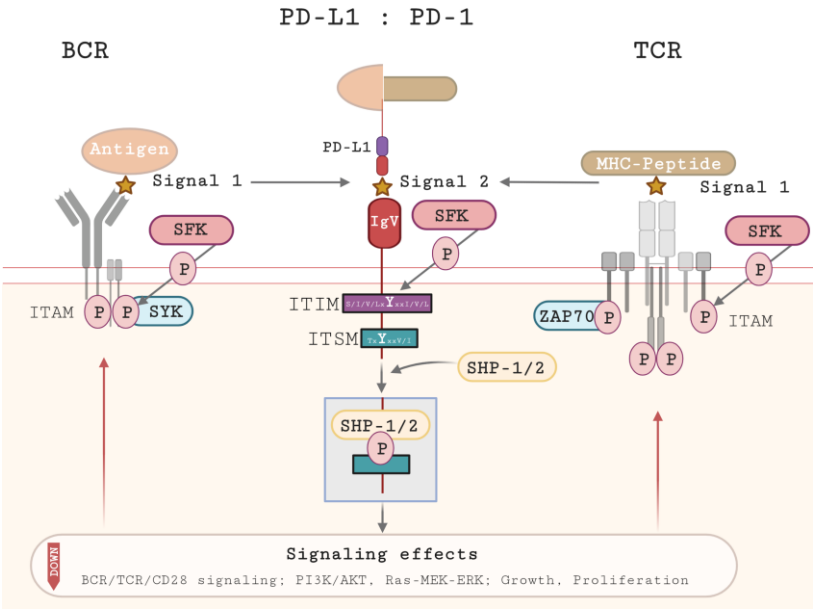


Figure 2.13 | PD-L1 : PD-1 signaling cascade

(i) Upon engagement of BCR/TCR (signal 1) SFKs are getting recruited, leading to phospho-ITAMs, which recruit cell-dependent SYKs to start activating signaling cascade. (ii) PD-L1:PD-1 ligation (signal 2) in proximity to signal 1 leads to tyrosine residue phosphorylation of PD-1 ITIM and ITSM by SFK Lyn. (iii) Recruitment of tyrosine phosphatases SHP-1 and SHP-2, initiating downstream signaling, thereby inhibiting events caused by signal 1 events. (Adapted from (Chinai, Janakiram et al. 2015).)

The downstream signaling effects of PD-L1:PD-1 engagement are generally of inhibitory nature, such as SHP-mediated dephosphorylation of protein kinases, thus limiting downstream signaling through PI3K/AKT, Ras-MEK-ERK or PLCγ2, as shown for B cells (Okazaki, Maeda et al. 2001, Patsoukis, Li et al. 2013). By this, signal transduction through calcium mobilization gets deactivated, culminating in growth retardation of B cells and T cells (Vibhakar, Juan et al. 1997, Latchman, Wood et al. 2001, Okazaki, Maeda et al. 2001). Additionally, other activating events are blocked by SHP-2, like CD28-mediated signaling, resulting in downregulation of the cell survival gene BCL-xL (Chemnitz, Parry et al. 2004). A more recent report indicates, that regulation of the costimulatory CD28 is the favored target, explaining PD-L1:PD-1 axis function outside B cells and T cells and highlighting the complex interaction network of the B7:CD28 superfamily (Hui, Cheung et al. 2017).

While the inhibitory effects of checkpoint signaling are well understood, potential activating events have been explored less. A feature of the ITSM in CD150 known to be responsible for switching regulating mechanism, is the ability to bind SH2D1A. However, PD-1 ITSM is not able to bind the adapter molecule, indicating towards other structural requirement to switch effector activity (Chemnitz, Parry et al. 2004).

2.4.3. Immune integrity

Given PD-L1s potency to be induced on a variety of cells, the PD-L1:PD-1 axis is responsible for regulation of many immune processes within the whole body. It is involved in immune cell development, as well as differentiation and plays a critical role in establishment of peripheral tolerance.

Lymphocyte development and Central Tolerance

PD-1 expression was found in adult bone marrow pro-B cells and inducible with IL-17, a cytokine dispensable for cell lineage development of human fetal bone marrow pro-B cells (Finger, Pu et al. 1997). For T cell lineage development, PD-1 is similarly important: In the DN T cell precursor population, high percentage of PD-1 positive cells was found, compared with other thymocyte population, hinting towards a role in the selection process (Nishimura, Agata et al. 1996). In accordance with this, PD-1-deficient mice exhibited a selective increase of DP population but reduced efficiency for certain $CD8^+$ subsets, pointing towards a role in both, positive and negative selection (Nishimura, Honjo et al. 2000). However, another PD-1-deficient mouse model showed no difference in DP T cell number compared to wildtype, but a significant activation and proliferation of autoreactive $CD8^+$ T cells, highlighting the role in establishing peripheral tolerance (Nishimura, Nose et al. 1999).

Peripheral tolerance

Given PD-1s role in peripheral tolerance, mice deficient for PD-1 developed a range of autoimmune disease. PD-1-deficient mice on the C57BL/6 background were shown to develop lupus-like proliferative arthritis and glomerulonephritis with IgG antibody increase by 14 months (Nishimura, Nose et al. 1999). Consistent with this, BALB/c–PD-$1^{-/-}$ mice developed dilated cardiomyopathy, accompanied with

deposition of IgG within the heart and the presence of autoantibodies specific for heart tissue (Nishimura, Okazaki et al. 2001). Furthermore, PD-1 deficient mice developed within 10 weeks of age multiorgan lesions histologically comparable to graft-versus-host disease, showing that autoreactive peripheral T cells can be activated in the absence of PD-1 (Nishimura, Nose et al. 1999).

Allogenic response

Very strong PD-L1 expression was found in placenta linking it to feto-maternal tolerance by inhibition of the maternal immune system and thereby down-regulating allogenic T cell response (Brown, Dorfman et al. 2003). This role was strengthened when pregnant PD-L1 deficient mice or pregnant mice treated with anti-PD-L1 mAb promoted expansion of alloreactive Th1 cells which led to an increase in fetal rejection (Guleria , Khosroshahi et al. 2005). Equally important is PD-L1 expression after transplantation. As shown, when PD-L1 KO liver grafts were transplanted into WT mice, leading to increased necrosis levels (Ueki, Castellaneta et al. 2011). Administration of PD-L1 antagonizing antibody in several other transplant models confirmed the key role in rejection of transplanted tissues (Ito, Ueno et al. 2005, Hori, Wang et al. 2006).

Infectious disease

Several studies highlighted the pivotal role of the PD-L1:PD-1 pathway for infection in balancing effective antimicrobial defense and inflammation-associated tissue damage. Adenovirus infected PD-1 deficient mice clear infection more rapidly but with the consequence of more severe hepatocellular injury compared to infected WT mice (Iwai, Terawaki et al. 2003). The administration of antagonistic PD-L1 antibody to a mouse model for herpetic stromal keratitis led to a decrease in herpes simplex virus-specific $CD4^+$ T cells, resulting in an exaggerated pathogenesis (Jun, Seo et al. 2005). Linked with activation of the pathway are, apart from several viral infection, chronically infecting bacteria, such as *H. pylori* (Das, Suarez et al. 2006), parasitic worms like *Schistosoma mansonii* (Smith, Walsh et al. 2004) and protozoan parasites such as *Leishmania sp*. (Liang, Greenwald et al. 2006). Chronic infections are characterized by T cells, that are persistently exposed to antigens or inflammation, leading to the development of ‘exhausted’ $CD8^+$ T cells. These cells

have lost effector functions, such as proliferation, cytokine production and degranulation. Accompanied is T cell exhaustion by expression of inhibitory molecules, such as PD-1 (Barber, Wherry et al. 2006, Wherry, Ha et al. 2007). By this, control of infection is prevented as PD-L1/PD-1 antagonizing treatment is able to restore T cell function and reduce virus load (Day, Kaufmann et al. 2006, Zhang, Zhang et al. 2007). The presence of exhausted T cells, resulting in an impaired immune surveillance, can give rise to the development of cancer.

2.4.4. Tumor immunity

As the PD-L1:PD-1 signaling axis was shown to be involved in various immune processes, aberrant expression can likewise display severe consequences. Cells escaping from immune surveillance are able to accumulate mutation and progress towards cancer. PD-L1 was demonstrated to enhance immune evasion by increase of epithelial-mesenchymal transition and be a prerequisite for the development of cancer, as shown for skin cancer (Cao, Zhang et al. 2011). T cells infiltrating human tumors and peripheral T cells, targeting tumor-specific mutant neoantigens were likewise marked by PD-1 presence, highlighting the role in immune invasion (Gros, Robbins et al. 2014, Gros, Parkhurst et al. 2016). Cancerous cells of hemopoietic and nonhemopoietic origin are characterized by increased PD-L1 expression (Yamazaki, Akiba et al. 2002). Additionally, a study suggested, that on cancer cells, PD-L1 can act as receptor, transmitting antiapoptotic signals which result in resistance to $CD8^+$ Tc cell cytolysis and Fas- or drug-induced apoptosis (Azuma, Yao et al. 2008).

Immune checkpoint inhibition

In 2002, Tasuku Honjos group postulated that in tumor cells the PD-1:PD-L1 signaling pathway acts as escaping mechanism from the host T cell immunity and raised for the first time the possibility of a pathway blockage as tumor therapy (Iwai, Ishida et al. 2002). Since then, many drugs targeting this pathway have successfully been tested (Table 2-2), giving hope to advanced stage cancer patients, as shown for melanoma patients, where treatment with the anti-PD-1 antibody lambrolizumab resulted in sustained tumor regression (Hamid, Robert et al. 2013). In more and

more cancers immune checkpoint inhibition is becoming a central pillar of treatment strategies.

Table 2-2| Immune checkpoint inhibitors targeting PD-L1 : PD-1 axis

	Name	Indication
PD-1	Lambrolizumab	Melanoma (Hamid, Robert et al. 2013)
	Nivolumab	Melanoma, NSCLC, SCLC, RCC, HCC, Hodgkin lymphoma, head and neck cancer, urothelial carcinoma, microsatellite instability-high, or mismatch repair-deficient metastatic colorectal cancer
	Pembrolizumab	Melanoma, NSCLC, head and neck squamous cell carcinoma, Hodgkin lymphoma, urothelial carcinoma, microsatellite instability-high cancer, gastric cancer, cervical cancer, primary mediastinal large B-cell lymphoma
PD-L1	Atezolizumab	NSCLC, urothelial carcinoma
	Durvalumab	NSCLC, urothelial carcinoma
	Avelumab	Urothelial carcinoma, Merkel cell carcinoma

Adapted from (Tajiri and Ieda 2019)

In 2018 the discovery of immune checkpoint therapy was acknowledged by awarding James P. Allison and Tasuko Honjo with the Nobel Prize in Physiology for their fundamental work in this field (Nobelprize.org 2018).

2.5. [E] The gastric pathogen *Helicobacter pylori*

The diversity of microbial communities is the reason for the existence of multicellular organism and since then, animals harbor microbes performing cooperative interactions, including defense, metabolism and reproduction. As shown by phylogenetic studies, even before humans migrated from Africa, the Gram-negative bacterium *Helicobacter pylori* / *H. pylori* has been associated with them and still colonizes over half of the world's population (Parsonnet 1995, Covacci, Falkow et al. 1997), making it one of the most successful bacteria in the human body. In 1984, Barry Marshall and Robin Warren discovered the "bacilli in the stomach of patients with gastritis" and showed one year later, by performing experiments on themselves, that it can act as a pathogen (Marshall and Warren 1984, Marshall, Armstrong et al. 1985). Prevalence of infection with *H. pylori* varies worldwide and correlates with lower socioeconomic conditions from 10% to greater than 80% (Hunt, Xiao et al. 2011, Queiroz, Harris et al. 2013). Transmission of *H. pylori* occurs largely by oral-oral or fecal-oral routes with infection being usually acquired during the first decade of life (Malaty, El-Kasabany et al. 2002, Hunt, Xiao et al. 2011). Several unique strategies enable the bacterium to colonize the harsh environment of the human stomach and to persist by immune evasion. Course and outcome of infection differ among strains and depend on environmental parameters, host genetic susceptibility conditions and bacterial factors.

2.5.1. Reaching the gastric epithelial cell

H. pylori is a gram-negative helicoidal shaped bacteria, measuring 2.4 – 4.0 µm in length and 0.5 – 1.0 µm in width. It is mostly found in the antrum, the lower part of the human stomach (Hooi, Lai et al. 2017). The environment in the gastric lumen is highly acidic with pH 6 to pH 2. Still, for about half an hour *H. pylori* is able to survive there by producing urease and thereby neutralizing pH (Schreiber, Bücker et al. 2005). With around 80%, the majority of bacteria are found in the mucus, living in microcolonies (Hessey, Spencer et al. 1990, Hidaka, Ota et al. 2001). However, a small population directly interacts with gastric cells, playing the pivotal role in pathogenesis. To reach the gastric epithelium, where the pH is close to neutral, a

thick mucus lining needs to be penetrated. Mucin viscoelasticity increases with acidity and becomes less gel-like due to urease activity (Celli, Turner et al. 2009). The helical shape enables the bacterium to move in a cork-screwing motion (Sycuro, Wyckoff et al. 2012). Promoted by two to six flagella, chemotactic receptors and pH-dependent adherence enable *H. pylori* to attach (Censini, Lange et al. 1996). Of special importance are the over 50 β-barrel outer membrane particles/OMPs, which are divided into five major families and serve as porins, iron transporters and adhesins (Alm, Bina et al. 2000). Among them, the pH-controlled 'blood group antigen-binding adhesin A'/BabA, binding to fucosylated structures, such as the Lewis B/Le^b blood group antigen (Boren, Falk et al. 1993, Ilver, Arnqvist et al. 1998). These antigens are abundant on gastric epithelial cells and mucins, especially MUC5AC, which is secreted by gastric epithelial cells to the apical surface (Bugaytsova, Björnham et al. 2017, Gonciarz, Walencka et al. 2019). BabA-binding to Le^b is reversible and pH dependent, which protects *H. pylori* of epithelial cell turnover, peristalsis and gastric emptying without being shed into the acidic bactericidal lumen. It further marks the preferences for the antrum, as no acid-secreting parietal cells are found there and the mucus layer is thicker, so more protective against acids (Bugaytsova, Björnham et al. 2017). Other OMPs involved in direct adherence with the host cell are AlpA/B, SabA, OipA, HopQ (Matsuo, Kido et al. 2017). All exhibiting affinity towards distinct interaction partners, such as HopQ with affinity for the immunoglobulin 'carcinoembryonic antigen-related cell adhesion molecule'/CEACAM superfamily, thereby promoting binding to the epithelium (Belogolova, Bauer et al. 2013).

The process, how *H.pylori* reached the gastric epithelial lining is illustrated in the figure below (Figure 2.14).

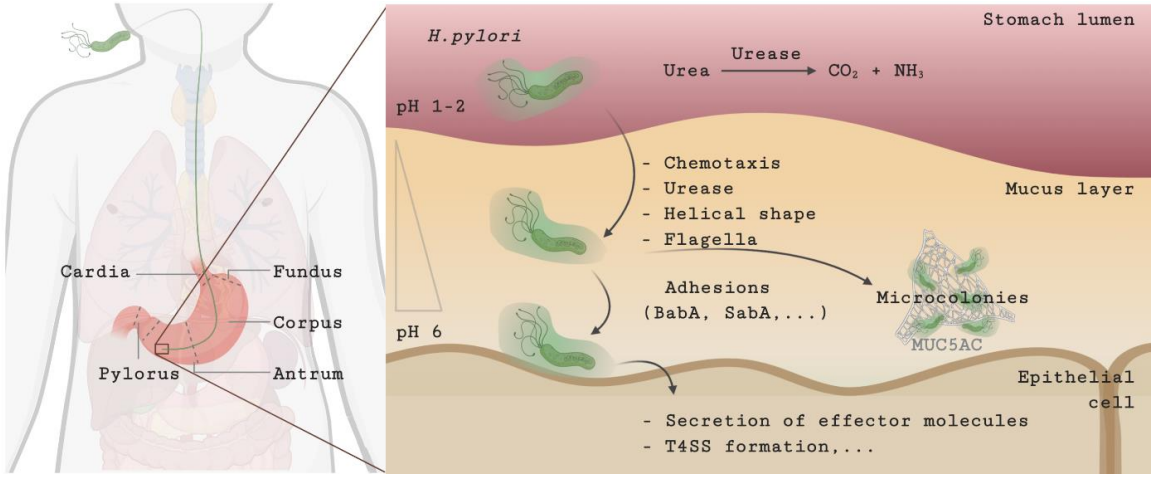


Figure 2.14 | Initial colonization

Through oral-oral or fecal-oral routes, *H. pylori* infection leads to colonization primarily of the stomach antral region. Escape from the acidic stomach lumen towards the close to neutral pH epithelial lining is chemotaxis driven. Urease-dependent ammonia production locally raises the pH, allowing *H. pylori* to shortly withstand acidic environment and lower viscoelasticity of mucus layer. Helical shape and flagella promote movement. Adhesion molecules enable binding, such as BabA to Le^b, which is present on MUC5AC and gastric cells. Approximately 80% of *H. pylori* are found within the mucus in predominantly MUC5AC-rich regions, forming microcolonies. Proximity and subsequent adhesion to epithelial cells, enable release of effector molecule, of which some are directly translocated via type 4 secretion system into the host. (Adapted from (Salama, Hartung et al. 2013).)

2.5.2. Colonization through virulence factors

Close proximity to the gastric epithelium is pivotal for *H. pylori* to establish its microniche through either direct secretion of virulence factors or via formation of a secretion system. Accordingly, adhesions like BabA and HopQ, essential for the initial colonization phase, were shown to be indispensable for effector molecule transport via the secretion system (Ishijima, Suzuki et al. 2011, Belogolova, Bauer et al. 2013). Among *H. pylori* strains, presence and activity of virulence factors differs, affecting the outcome of the infection. A first classification was based on disease severity with bacterial isolates from patients suffering of severe gastritis or gastroduodenal ulcers. These strains, labeled as type I-strains are marked by the activity of a cytotoxin named VacA and the presence of a toxin-associated antigen named CagA (Xiang, Censini et al. 1995).

Vacuolating toxin

The vacuolating toxin VacA was originally found to be secreted by *H pylori* strains, isolated from patients with peptic ulcer disease, leading to vacuolization in a variety of cells, including epithelial cells and T cells (Cover and Blaser 1992). Later on, it

was found to be present in all strains, however with varying activity, which depends on gene structure and is associated with more severe clinical consequences (Atherton, Cao et al. 1995). Release of VacA near the apical membrane leads to membrane binding and pore formation upon oligomerization in lipid rafts, resulting in the endocytic internalization of the toxin. There, anion-selective channels are formed in membranes of endocytic compartments leading to swelling, thus vacuolization (Cover and Blanke 2005). Apart from this, secretion of VacA leads to numerous responses on susceptible cells, such as apoptosis by cytochrome c release from mitochondria, inhibition of cell growth and the ability to modulate inflammatory response (reviewed in (Yamaoka 2010)).

cag pathogenicity island

The second virulence determining-factor of *H. pylori* type-I strains is the presence of CagA, which is encoded on the cag pathogenicity island/cagPAI (Censini, Lange et al. 1996). About 31 genes are encoded on this highly conserved 40 kb island. Among them, approximately 20 genes encode proteins, forming a needle-like surface appendage, a type IV secretion system (T4SS) which emerges from the bacterial surface to translocate molecules into host cells (Censini, Lange et al. 1996, Akopyants, Clifton et al. 1998). Tight bacterial adherence to the host cell through adhesions, such as the cagPAI encoded protein CagL, which interacts with host integrins, leads to T4SS formation and subsequent secretion of effector molecules. The best characterized factor, translocated via cagT4SS, is the 'cytotoxin-associated gene A'/CagA. It is present in approximately 60% of infections in western countries and strains harboring the gene, cause a more severe disease outcome (Covacci, Censini et al. 1993, Tummuru, Cover et al. 1993, Blaser, Perez-Perez et al. 1995). The highly polymorphic gene *cagA* differs in numbers of C-terminal regions, containing the Glu-Pro-Ile-Tyr-Ala (EPIYA) motif which corresponds with disease severity (reviewed in (Hatakeyama 2014)). The tyrosine residue within the motif gets phosphorylated by SFKs and c-Abl tyrosine kinases upon translocation. Dependent of this, cytoskeletal rearrangement leads to formation of the *in vitro* hummingbird phenotype. Over 20 proteins were identified to interact with translocated CagA in both, phosphorylation dependent and

independent manner, leading to cellular elongation, defects in polarity and motility, antiapoptotic response through p53 degradation and a proinflammatory response. The *in vivo* pathobiological potential of CagA was validated in numerous epidemiological studies and in mice, expressing transgenic CagA, all showing an association with greater inflammatory severity and development of gastric diseases, such as epithelial hypertrophy or gastrointestinal tumors (Takahashi-Kanemitsu, Knight et al. 2020). Apart from CagA, peptidoglycans, bacterial DNA and the LPS metabolite HBP were found to be translocated via cagT4SS, leading to *H. pylori*-evoked exacerbated disease severity by enhanced proinflammatory response (Viala, Chaput et al. 2004, Suarez, Romero-Gallo et al. 2015, Varga, Shaffer et al. 2016, Zimmermann, Pfannkuch et al. 2017).

2.5.3. Persistence by immune evasion strategies

H. pylori shelters from the acidic environment of the stomach lumen through the mucus lining towards the epithelial lining. Although capable to transmigrate towards the submucosa, it is predominantly found apically (Keilberg, Zavros et al. 2016, Tegtmeyer, Wessler et al. 2017). As lymphocytes reside in the lamina propria, first immune effectors such as antimicrobial peptides and secreted antibodies, are located on the apical side. Investigated gastric biopsies of *H. pylori* infected patients revealed upregulation of several antimicrobial peptides, but with none providing sufficient activity to eradicate infection (Nuding, Gersemann et al. 2013). Human beta defensin 3, which exhibits strong antimicrobial activity is even downregulated in active chronic gastritis (Bauer, Wex et al. 2013).

Niche formation

It was shown, that cholesterol depletion activity, which is needed for bacterial membrane integrity, plays a pivotal role in the induction of an impaired immune response (Morey, Pfannkuch et al. 2018). A special feature to *H. pylori's* membrane is the presence of cholesteryl glucosides (Hirai, Haque et al. 1995). However, the bacterium lacks enzymes for *de novo* synthesis of sterols. To overcome this, it exhibits chemoattraction towards cholesterol and binds to cholesterol found in the medium or present in host cell lipid (Wunder, Churin et al. 2006). Binding with

phosphatidylethanolamine, leads to glycosylation by the membrane-bound enzyme cholesterol-α-glucosyltransferase (CGT). Cholesterol gets further modified until it can be incorporated into the bacterial membrane (Lebrun, Wunder et al. 2006). By this process the host cell membrane is depleted of cholesterol. As cholesterol is an integral part of host membrane lipid rafts, where various receptors are assembled, disruption leads to decrease in signaling ability, as it was demonstrated for IFNγ response upon infection. This mechanism leads to formation of a microniche for *H. pylori*, providing protection from immune signaling through an impaired responsiveness of the cell (Morey, Pfannkuch et al. 2018). Another niche forming strategy was observed in short-term infection of primary gastric epithelial mucusoids, where mucus with antimicrobial factors is produced, able to avoid colonization of other *H.pylori* strains, thereby stabilizing the existent infection (Boccellato, Woelffling et al. 2019).

PAMP modification

In order to respond to infection, cells use a range of PRRs, triggering inflammatory response to clear infection. However, *H. pylori* largely avoids immune recognition. Detection of *H. pylori* derived LPS by TLR4 is avoided through modification of the hydrophobic lipid A domain, which anchors LPS to the bacterial outer membrane. Modifications include low level phosphorylation, aberrant acetylation pattern, increase in acyl chain length and the presence of Lewis antigens, leading to 1,000-fold lower endotoxicity as mediated by *Enterobacteriaceae* derived lipid A (reviewed in(Li, Liao et al. 2016)). Bacterial mutant strains, deficient for lipid A modificatory enzymes fail to colonize mice (Cullen, Giles et al. 2011). Likewise, *H. pylori* escapes flagellin recognition by TLR5 through modifications of the N-terminal TLR5 recognition domain (Andersen-Nissen, Smith et al. 2005).

Modulation of immune cell response

Though mostly found apically, *H. pylori* is also able to reside CagA-depend near tight junctions and to cleave them through secretion of the serine protease HtrA, thus able to directly modulate immune cells through bacterial virulence factors (Tan, Tompkins et al. 2009, Tegtmeyer, Wessler et al. 2017). VacA-dependent formation of anion-specific channels was shown to inhibit T cell activation and

proliferation thus inhibiting clonal expansion of immune cells which have previously been activated by *H. pylori* (Boncristiano, Paccani et al. 2003, Sundrud, Torres et al. 2004). Additional immunosuppressive factors inhibiting T cell proliferation were shown for the bacterial enzymes arginase and γ-glutamyl transpeptidase (Zabaleta, McGee et al. 2004, Schmees, Prinz et al. 2007). Other immune cells are modulated as well, including a cagPAI-driven inhibition of granulocyte migration (Busch, Weimer et al. 2015).

2.5.4. Clinical outcome of chronic infection

H. pylori is well enough adapted to co-exist in the human stomach for a human lifetime. About half of the world's population are infected with a highly variable clinical outcome (Figure 2.15). Most of the infections are asymptomatic, without a pathological phenotype. Spontaneous bacterial eradication occurs only in 1% of all cases (Lambert, Lin et al. 1995). Chronic infection can result in both, hypoacidity and hyperacidity, depending on the site of infection. Most chronically infected patients develop pangastritis, which is linked to reduction in acid production, a predisposition for gastric adenocarcinomas. Hyperacidity is linked with peptic ulcer disease (Smith, Hold et al. 2006). Population studies suggested protective effects of *H. pylori* induced acidity reduction in terms of esophageal conditions including gastroesophageal reflux disease, Barrett's oesophagus and even oesophageal adenocarcinoma (Corley, Kubo et al. 2008, Islami and Kamangar 2008, Sonnenberg, Lash et al. 2010, Sonnenberg, Turner et al. 2017). Similar correlations were found for inflammatory bowel disease (IBD) a chronic relapsing inflammation of the gastrointestinal tract, known to be a predisposing condition for colon cancer (Castano-Rodriguez, Kaakoush et al. 2017). *H. pylori* increases the risk for intestinal and diffuse type of gastric cancer (Plummer, Franceschi et al. 2015). Since 1994, it is classified as a group I carcinogen by the International Agency for Research on Cancer (IARC 1994, Logan 1994). In the model, proposed by Correa and co-workers, chronic gastritis can progress to atrophy which leads to intestinal metaplasia and subsequent gastric cancer over a course of 30 to 50 years (Correa, Haenszel et al. 1975). Gastric cancer is the 5th most frequent cancer with annually 876 000 new cases and 783 000 deaths, making it to the second leading cause of

deaths due to cancer.(Rawla and Barsouk 2019) As a hallmark of cancer, expression of immune inhibitory signals was observed for gastric cancer with over 40% of gastric carcinomas showing PD-L1 expression which was found to correlate with tumors size, depth of invasion, lymph node metastasis and survival time.(Wu, Zhu et al. 2006)

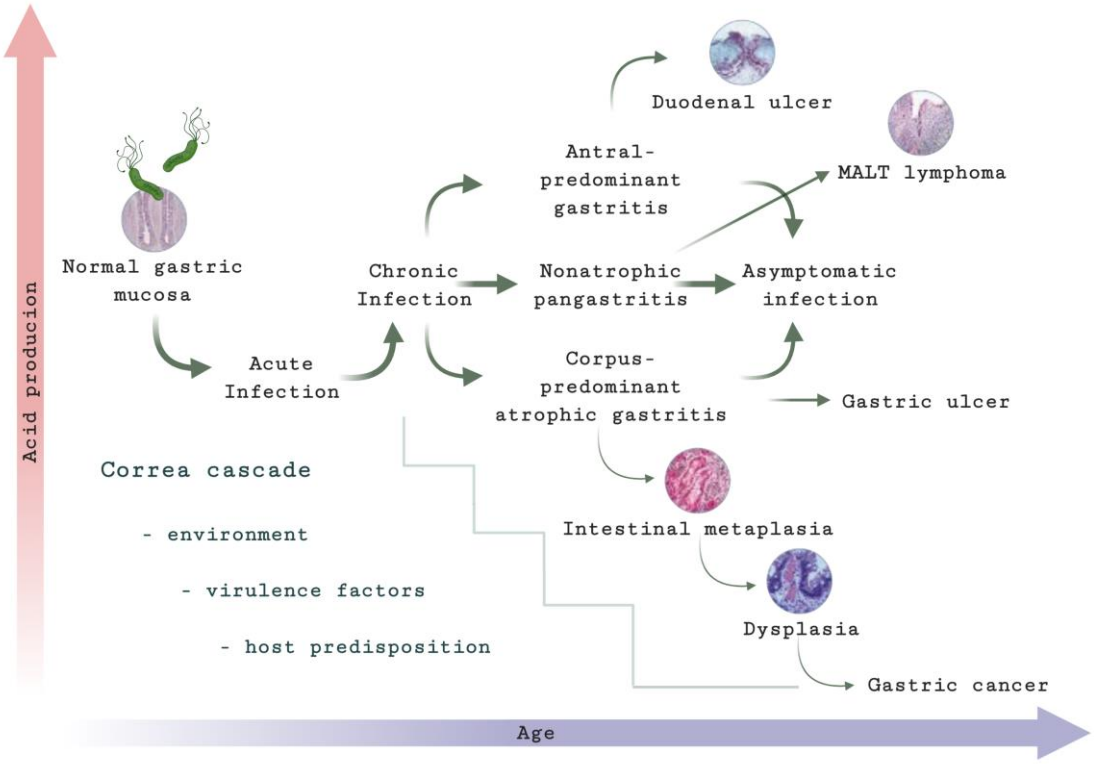


Figure 2.15 | Clinical outcome
Infection with the gastric bacterium *H. pylori* occurs usually in early childhood and lasts for a life time. Infection are characterized by gastritis with the majority of infection proceeding to be asymptomatic. Patients with high acid production level are more likely to suffer from gastritis predominant in the antrum, predisposing them to the development of duodenal ulcers. Persistent gastritis can lead to formation of aberrant B cell lymphoma, mucosa-associated lymphoid tissue (MALT). In contrast, patients with low level acid production are more likely to get corpus-predominant gastritis, a predisposition for gastric ulceration. *H. pylori* is a risk factor for the development of gastric cancer, which in the case of intestinal type GC is characterized by a series of precancerous lesions that have been shown to exacerbate the risk of GC. Development occurs in 1% of infected people and depends on environmental, bacterial and host factors. (Adapted from (Suerbaum and Michetti 2002))

Chapter

3. Aim of the thesis

The knowledge gap in *H. pylori*-driven PD-L1 induction

Infecting approximately 50 % of the worlds' population, the gastric bacterium *Helicobacter pylori* represents a special challenge to the human immune integrity (Parsonnet 1995). Infection is characterized by gastritis, which is usually not able to eradicate the pathogen, leading to chronic gastritis which can progress over atrophic gastritis, intestinal metaplasia and dysplasia towards gastric cancer (Correa, Haenszel et al. 1975) in 1 -3 % of the infected people (Peek and Crabtree 2006). During this cascade, a tightly regulated T cell population is majorly responsible for establishing immunological tolerance towards *H. pylori* (Ermak, Giannasca et al. 1998). An important regulator of T cell response is the immune checkpoint inhibitor programmed death ligand 1 (PD-L1) (Dong, Zhu et al. 1999, Freeman, Long et al. 2000, Dong, Strome et al. 2002). It is suggested to participate in the process of carcinogenesis, as in response to *H. pylori* exposure, expression of the ligand promotes a subset of non-proliferative regulatory T cells which suppress proliferation and cytokine production of other T cells, thereby protecting the pathogen and enabling its colonization (Beswick, Pinchuk et al. 2007). Furthermore, aberrant ligand expression occurs in a range of cancer types, including gastric cancer with up to 50 %, protecting the cancerous tissue from immune recognition (Böger, Behrens et al. 2016, Wang, Zhu et al. 2018, Liu, Choi et al. 2020).

Given PD-L1's role in bacterial colonization and gastric carcinogenesis, a better understanding of the mechanism leading to its upregulation is crucial. However, studies concerning causative underlying upstream effects are contradictory, indicating a gap in knowledge and possible treatment limitations (Das, Suarez et al. 2006, Lina, Alzahrani et al. 2015, Li, Xia et al. 2018). This thesis seeks to shrink this gap by delignating the regulatory mechanism in *H. pylori*-driven PD-L1 induction.
The gastric pathogen is equipped with a wide range of virulence factors to enable lifelong coexistence within the human body. Its infection process will be studied in detail and bacterial mutants, lacking specific factors will be used to narrow down the ligand activating signaling pathway. While initial experiments will be conducted in the easy-to-handle and low-cost gastric cancer cell line AGS, the more

sophisticated but also more expensive human gastric primary cell model is used for validation. With regard to possible treatments, inhibition or downregulation of involved host components will be evaluated for host integrity and microarray analysis will be used to investigate broader implications.

Chapter

4. Materials & Methods

4.1. General materials

4.1.1. Mammalian cell culture media

Table 4.1 | Cell culture media and supplements

Name	Supplier
Opti-MEM reduced serum media	Gibco, Life Technologies
Penicillin/streptomycin	PAN-Biotech
Puromycin	Gibco, Life Technologies
RPMI 1640 with L-glutamine and HEPES	Gibco, Life Technologies
FCS, heat inactivated	Biochrom
L-Glutamine	Invitrogen
Na-pyruvate	Gibco, Life Technologies
DMEM with 4.5 g/L D-glucose, NEAA	Gibco, Life Technologies
DPBS 1x	Gibco, Life Technologies

4.1.2. Plasmids

Table 4.2 | Plasmids

Name	Description	Application	Source
lentiCRISPRv2	CRISPR/Cas one vector plasmid system	CRISPR/Cas9 KO	Addgene
pMD2.G	Encodes for Vesicular stomatitis virus G glycoprotein (VSVG)	Lentiviral envelope vector	Addgene
psPAX2	Encodes for gag, pol, rev, and tat	Lentiviral packaging vector	Addgene

4.1.3. Buffers and solutions

Table 4.3 | buffers and solutions

Name	Component	Concentration
LB medium	NaCL	5 g/l
	Tryptone	10 g/l
	Yeast extract	5 g/l
PBS	NaCL	137 mM
	KCL	2.7 mM
	Na_2HPO_4	1.0 mM
	KH_2PO_4	1.8 mM
	pH adjusted to 7.4	
PBS-T	PBS	
	Tween 20	0.005 % (v/v)

4.1.4. Chemicals, consumables and commercial kits

Table 4.4 | Chemicals and reagents

Name	Supplier
DAPI	Roche
DNA loading dye 6x	Thermo Fisher Scientific
GeneRuler 1 kb plus DNA ladder	Thermo Fisher Scientific
HiperFect transfection reagent	Qiagen
LB Agar (Lennox Agar)	Invitrogen
LB Broth Base	Invitrogen
Lipofectamine 2000	Thermo Fisher Scientific
Mowiol 40-88	Sigma-Aldrich
Opti-MEM reduced serum media	Gibco, Life Technologies
PageRuler plus prestained protein ladder	Thermo Fisher Scientific
GeneJET RNA Purification Kit	Thermo Fisher Scientific
Plasmid Mini/Midi kit	Qiagen
Power SYBR Green RNA to CT 1-step Kit	Applied Biosystems
Hyperfilm ECL	Amersham Bioscience
Mini-PROTEAN TGX Gels, 4–15%	Bio-Rad Laboratories
Nitrocellulose membrane	Bio-Rad Laboratories
Polyvinylidendifluoridm (PVDF) membrane	Perkin Elmer

4.1.5. Enzymes

Table 4.5 | Enzymes

Name	Application	Supplier
*BsmB*I (FastDigest)	Restriction	Fermantas
*BamH*I	Restriction	Fermantas
Fast AP	Alk. phosphatase	Fermantas
Quick ligase	Ligation	New England Biolabs
T4 DNA ligase	Ligation	New England Biolabs
T4 polynucleotide kinase	Kinase	New England Biolabs

4.1.6. Laboratory instruments

Table 4.6 | Lab instrumentation

Name	Application	Supplier
CO_2 cell culture incubator	Cell culture	Binder GmbH
Fujifilm LAS-4000	Western blot	Fujifilm
Hera cell 150 Incubator	Cell culture	Thermo Fisher Scientific
Heraeus LaminAir	Microbiology	Thermo Fisher Scientific
Herasafe KS safety cabinet	Cell culture	Thermo Fisher Scientific

IX50-S8F inverted microscope	Microscopy	Olympus
Mini trans-blot cell	Western blot	Bio-Rad Laboratories
Mini-PROTEAN tetra cell	SDS-PAGE	Bio-Rad Laboratories
NanoDrop ND-1000 Spectrophotometer	Molecular biology	Thermo Fisher Scientific
StepOnePlus Real-Time PCR System	RT-qPCR	Applied Biosystems
TCS SP8	Confocal microscopy	Leica
Thermal cycler C100	PCR	Bio-Rad Laboratories

4.1.7. Software and databases

Table 4.7 | Software and databases.

Name	Application	Supplier
ChopChop	Prediction of CAS9 target sites	https://chopchop.rc.fas.harvard.edu/
Clone Manager v6	Cloning in silico	Sci-Ed Software
Excel 2010	Data processing	Microsoft
ImageJ	Image processing	Open source
NCBI	Biological databases	https://www.ncbi.nlm.nih.gov/
Prism v7	Data processing	GraphPad
ScanR Analysis	Image processing	Olympus
ScarnR Aquisition	Automated microscopy	Olympus
StepOne Software v2.3	qPCR analysis	Thermo Fisher Scientific
Biorender	Image compilation	https://biorender.com/
R	Data analysis, statistical analysis, data visualization	https://www.r-project.org/
R Studio	Data analysis, statistical analysis, data visualization	https://www.rstudio.com/

4.2. Cell culture and infection

4.2.1. Cell culture of adherent mammalian adherent cell lines

Cell lines

Most experiments were performed with cells, based on the human gastric cancer cell line AGS (Table 4.8), purchased at ATCC (CRL-1739) which originate from a 54 year old female person suffering from gastric adenocarcinoma.

Table 4.8 | Mammalian cell lines

Name	Description	MPIIB strain collection no.
AGS	Human gastric adenocarcinoma; PIV5-free	C-0579
AGS JAT003	AGS cells, stable expression of NF-κB-RE-luc2P	C-0693
AGS LAM004	AGS ALPK1-/- cells	C-0741
AGS JAB004	AGS HK2 $^{-/-}$ cells	C-0739

Cultivation

Cultures were kept at 37°C in a humidified atmosphere with 5% CO_2 and, unless indicated otherwise, all cell lines were cultivated in RPMI supplemented with 10 % fetal calf serum (FCS). When cells reached confluency of 90%, they were subcultured. To split them, they were washed twice with DPBS to get rid of the FCS which would abolish the activity of trypsin, that was added afterwards. Following 5 min of incubation at 37°C, cells were reseeded to reach an initial confluency of 30 %. For experiments, only cells between passage 2 and 10 and with a confluency of 60-70% were used.

4.2.2. Cell culture of primary human gastric cells

The following methods are adapted from (Schlaermann, Toelle et al. 2016).

Material

Primary cells were isolated from human gastric tissue material, which was obtained from the Charité Centre for Obesity and Metabolic Surgery located in Charité University Medicine, Berlin, Germany and the Clinic for General, Visceral, and Oncologic Surgery, department of Obesity and Metabolic Surgery in the Helios Clinic, Berlin-Buch. The tissue was removed from the surgeon during gastric sleeve resection and originated from the greater curvature of the human stomach. The Ethics Commission of the Charité, Berlin (EA1/129/12) approved the scientific

usage of the gastric material. The tissue material was stored in ChillProtect buffer until further processing. The used human material is indicated in the following table (Table 4.9).

Table 4.9 | Primary human material

Name	Sex	Age	Region
hGAT18	f	50	Antrum
hGAT23	f	55	Antrum
hGAT24	m	47	Antrum

Isolation

A small piece of human tissue sample (approximately 2 x 2 cm) identified as antrum specimen, was cleaned by at least three rounds of washing with cold PBS supplemented with 1.25 µg/ml Amphotericin, 50 µg/ml Gentamycin and 100 U/ml Streptomycin/Penicillin. After cleaning the tissue sample from adipose tissue muscles and blood vessels, it was minced into small pieces (1 mm^3), transferred into a fresh 50 ml-falcon tube and washed again with PBS (25 ml) by vortexing. The tissue pieces were left to precipitate and the step was repeated until the supernatant was clear. Under constant slow agitation, the pieces were incubated with 45 ml chelating solution for 20 min at 37°C. A sterile petri dish was used for the obtained clean tissue pieces. A sterile glass slide was utilized to gently squeeze the tissue pieces in order to obtain gastric glands. Using 3 ml cold Adv+++, extracted glands and tissue pieces were collected in a falcon. Force via pipetting up and down was applied to release of further gastric glands and eventually epithelial cells. The supernatant was collected after 3 min in which tissue pieces settled due to gravity. The washing step was repeated for five times. From the final cell suspension, 10 µl were microscopically analyzed to assess the cell number. Subsequently, the suspension was centrifuged with 300 x g for 5 min at 4°C and washed three times with cold Adv++. The supernatant was discarded and cells were resuspended in Matrigel on ice. A drop of 50 µl Matrigel-cell mixture, containing about 300 gastric glands, was placed per well in a 24-well plate and subsequently incubated for 30 min at 37°C without addition of medium. Upon

Matrigel solidification, 500 µl of 3D medium (Table 4.10) were added per well and cultured at 37°C in a humidified atmosphere with 5% CO_2.

Passaging

To subculture organoids, Matrigel had to be thawed in ice and a new, sterile 24-well plate had to be pre-warmed in the incubator. Supernatant of cells was removed. The Matrigel drop was resolved by pipetting 1 ml of cold Adv++ up and down and collected, together with up to five additional well, within on 15 ml falcon. Cells were centrifuged with 300 x g for 5 min at 4°C. The resulting cell pellet was resuspended in 1 ml TryLE and incubated for 10 min at 37°C and vortexed several times during the incubation. To obtain single cells, a narrow Pasteur pipette was used afterwards and number of single cells was microscopically assessed. To inactivate TrypLE, 6 ml of the FCS-containing reagent Adv+++ was added. Cells were centrifuged with 300 g, for 5 min and the resulting pellet was washed with Adv++, following another round of centrifugation. The cell pellet was resuspended in Matrigel with 30 000 cell/50 µl and Matrigel drops were placed in the initially pre-warmed 24 well plate. The plate was placed in the incubator for 30 min and 500 µl 3D medium (Table 4.10) was added after Matrigel polymerization to be stored at 37°C, 5 % CO_2. The ROCK inhibitor concentration of 2.5 µl/ml of freshly seeded cells was reduced to 0.5 µl/ml after the first three days of cultivation. Every third day, medium was changed.

2D cultivation

In contrast to the 3D culture, which contains antibiotics in the Matrigel and mostly resembles non-differentiated cells, the planar 2D culture represents a convenient model for experimental approaches. Initially, a 24-well plate was collagen coated with a mixture of 1.5 %(v/v) bovine collagen with 0.02 M acetic acid. The solution was incubated for 10 min at 37°C. Afterwards, the plate was dried under sterile conditions. To transfer organoids into a monolayer culture, samples were prepared similarly to the passaging procedure. However, cells were not resuspended in Matrigel but in 2D culture medium to be able to seed 2.5 10^5 cells in 500 µl of 2D medium (Table 4.10). In contrast to organoids from the 3D culture, 2D cells

gene expression pattern is highly similar to gene expression, obtained in differentiated gastric organoid.

Table 4.10 | Primary human medium

Supplements	2D medium	3D medium
WNT3A conditioned medium	25 %	50 %
R-SPO1 conditioned medium	12.5 %	25 %
ADF ++	ad 100%	ad 100%
FCS, heat inactivated	5 %	-
B27	2 %	2 %
Nicotinamide	15 mM	10 mM
N2	1 %	1 %
Penicillin/Streptomycin	-	100/100 U/ml
N-Acetylcysteine	-	1.25 mM
Human EGF	20 ng/ml	20 ng/ml
Human FGF-10	150 ng/ml	150 ng/ml
Human Noggin	150 ng/ml	0.15 mM
Human (Leu-) Gastrin	10 nM	10 nM
Y-27632 (ROCK-inhibitor)	3 µM	7.5 µM/1.5 µM (*)
A-83-01 (TGFβ-inhibitor)	0.25 µM	1 µM

(*) reduced to 1.5 µM three days post shearing

4.2.3. Culture and in vitro infection of *Helicobacter pylori*

Bacterial strains

The *Helicobacter pylori* P12 wildtype strain served as standard in this study. Infection with bacterial mutants (Table 4.11) was performed to investigate the dependency of virulence factors.

Table 4.11 | Bacterial strains

Name	MBIIP strain collection number	Antibiotic resistance
H.p. P12 WT	P511	vancomycin
H.p. P12 Δ*cagA*	P378	vancomycin + chloramphenicol
H.p. P12 Δ*cagPAI*	P387	vancomycin + kanamycin
H.p. P12 Δ*cgt*	P451	vancomycin + chloramphenicol
H.p. P12 Δg*mhA*	P585	vancomycin + kanamycin
H.p. P12 Δg*mhB*	P586	vancomycin + kanamycin
H.p. Δ*rfaE*	P588	vancomycin + kanamycin
H.p. ΔvacA	P216	vancomycin + chloramphenicol

Cultivation

To store *H.pylori*, bacterial aliquots are frozen at -80°C in glycerol freezing medium. For every experiment, a new aliquot had to be thawed. Bacteria were plated out on GC agar plates (Table 4.12) containing the strain-specific antibiotics and kept under microaerophilic conditions (5 % CO_2, 4 % O_2) at 37°C for three days. Then, *H.pylori* were overplated on a fresh plate and incubated overnight. At this point, bacteria will be prepared for infection.

Table 4.12 | GC agar composition

Supplement	concentration
Gonococci (GC) Agar in H_2O	36 mg/ml
Horse serum, inactivated	10 %
Vitamin-Mix	1 %
Nystatin:	1 µg/ml
Trimetoprim:	5 µg/ml
Vancomycin	10 µg/ml

Infection

Prior to infection, mammalian cells were starved (3 h for cell lines and overnight for primary cells) with serum-free cell culture medium. One day before infection, serial dilutions of *H. pylori* were plated on the respective GC agar plates. On the day of infection, bacteria were harvested with a cotton swap, resuspended in serum-cell culture medium and the optical density $(OD)_{550}$ was measured to determine the number of bacteria. Calculations are based on a standard curve (Suppl.Figure 9.1) which was generated within the department by blotting the OD_{550} values of a serial, pre-defined *H. pylori* dilution against the colony forming units per ml (CFU/ml). To determine the multiplicity of infection (MOI), number of mammalian cells was calculated based on the size of the wells, the confluency of the cells and number of cell, counted using a hemocytometer. Supernatant of mammalian cells was discard and the calculated bacterial volume was added. Bacterial fitness was assessed by eye (bacteria layer on plates has to look rather yellow than brown) and microscopically 30 min post infection (bacteria should move fast).

4.3. Molecular techniques

4.3.1. Polymerase Chain Reaction

The Polymerase chain reaction (PCR) is a technique to amplify DNA fragments. It is based on DNA polymerase activity to elongate short, predefined oligonucleotides (primer, Table 4.13) using a DNA template strand. It is a three step procedure: (1) A DNA denaturation cycle, (2) upon which primers anneal and (3) an elongation step, in which the primed DNA template gets extended by DNA polymerase. Following, the reaction mix (Table 4.14) and thermocycler setup (Table 4.15) are indicated.

Table 4.13 | Primer for PCR

Gene		Sequence 5'-3'
HK2	Forward	CAT CTG CTT GCC TAC TTC TTC A
	Reverse	AAA CTT TTT CCG CTC CCA G

Table 4.14 | Master mix for PCR

Compound	Volume
Plasmid DNA	X µl (200 ng)
5x PCR GC buffer	10 µl
dNTP mix (10mM)	1 µl
Forward primer (100µM):	1 µl
Reverse primer (100µM):	1 µl
Phusion DNA Polymerase	0.5 µl

Table 4.15 | Program setting for PCR

	Duration	Temperature	
Polymerase activation	1 min	98°C	1 cycle
Denaturation	10 s	98°C	35 cycles
Primer annealing	30 min	59°C	
Elongation	2 min	72	

4.3.2. qRT-PCR

Quantitative real-time PCR is a fluorescence-based technique to measure amounts of RNA. The starting material is RNA which gets transcribed into complementary

DNA (cDNA) by activity of reverse transcriptase. Then, cDNA is used as template for the subsequent RT-qPCR. Here, the one-step method was used, in which reverse transcription and PCR are combined in a single tube and buffer, containing both enzymes, transcriptase and DNA polymerase

Cell preparation

Medium of the cells from a 24 well plate was aspired and 300 µl lysis buffer (GeneJET RNA Purification Kit) was directly added into the wells. The plate was frozen for at least 1h at -80°C to allow further tissue disruption. Then, the cell suspension was thawed and 150 µl of 96% (v/v) ethanol were added and RNA was extracted by applying a silica-based membrane technology, following the manufacturers' protocol (GeneJET RNA Purification Kit). To analyze the extracted RNA, 1.5 µl of the sample were measured using a NanoDrop ND-1000 Spectrophotometer. Due to the aromatic ring within the RNA structure moieties purine and pyrimidine, absorbance around 260 nm is RNA specific. In contrast aromatic amino acids show absorbance near 280 nm. To determine the purity of the samples, the 260/280 ratio was used with a ratio around 1.8 being considered pure.

Experimental set-up

The Power SYBR Green RNA-to-CT 1-Step Kit was used according to the manufacturer with reaction scaled to 25 µl and the mixture was prepared as indicated below (Table 4.16) using gene specific forward and reverse primers (Table 4.17).

Table 4.16 | Master mix for RT-qPCR

Compound	Volume
2x Power SYBR® Green RT-PCR	12.5 µl
RT enzyme Mix	0.2 µl
Forward Primer (10 µM)	0.25 µl
Reverse Primer (10 µM)	0.25 µl
RNA sample adjusted to 1 µg/µl	10 µl
ddH_20	1.8 µl

Table 4.17 | Primer for RT-qPCR

Gene		Sequence 5'-3'
IL-8	Forward	ACA CTG CGC CAA CAC AGA AAT
	Reverse	ATT GCA TCT GGC AAC CCT ACA
HK1	Forward	CTG CTG GTG AAA ATC CGT AGT GG
	Reverse	GTC CAA GAA GTC AGA GAT GCA GG
HK2	Forward	CAA AGT GAC AGT GGG TGT GG
	Reverse	GCC AGG TCC TTC ACT GTC TC
PD-L1	Forward	GGC ATT TGC TGA ACG CAT
	Reverse	CAA TTA GTG CAG CCA GGT

All primers were purchased from metabion international AG

The StepOnePlus Real-Time PCR System was used to perform the RT-qPCR with the conditions below (Table 4.18). Within the initial cycle, the RNA gets transcribed into cDNA using reverse transcriptase. During the second step, the *Taq* polymerase gets activated. This is followed by 40 cycles of denaturation, followed annealing and elongation.

Table 4.18 | Program setting for qRT-PCR

	Duration	Temperature	
Reverse transcription	30 min	48°C	1 cyle
Polymerase activation	10 min	95°C	1 cycle
Denaturation	15 s	95°C	40 cycles
Primer annealing	1 min	60°C	

After completing the 40 cycles, a ramp speed of 0.3 % between 60°C and 95°C was applied to determine a melting curve to confirm gene-specific amplification. StepOnePlus Real-Time PCR System software was used to determine baseline and Cq values. Fold-changes were calculated by applying the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). Results were normalized to the house-keeping gene GAPDH and the untreated/uninfected experimental condition.

4.3.3. DNA restriction and ligation

A restriction enzyme is a protein, recognizing a specific short (4-8 bp) DNA motif on double stranded DNA and catalyze its cleavage by hydrolysis of phosphodiester bonds. Depending on the restriction enzymes, either sticky or blunt DNA fragment ends are produced in the digestion process. Restriction enzymes are widely used for generation of recombinant DNA and the verification of DNA sequences. The reaction mix (Table 4.19) was pipetted and incubated for 1 h at 37°C.

Table 4.19 | Master mix for DNA restriction

Compound	Volume
Plasmid DNA	x µl (0,5 µg)
Restriction enzyme I	0.5 µl
Restriction enzyme II	0.5 µl
10x Reaction buffer	2.5 µl
ddH_20	ad 25 µl
Plasmid DNA	x µl (0,5 µg)

4.3.4. Transformation of *E. coli*

The transformation process is used to introduce foreign DNA into bacteria cells. Within this thesis, plentiCRISPRv2 plasmid DNA was introduced into the chemically competent cell line Stbl (Invitrogen). In detail, 50 µl of bacteria were slowly thawed on ice and incubated with 10 µl plasmid ligation mix for 20 min at 4°C, following a heat-shock for 30 s at 42°C and an additional incubation for 2 min at 4°C. To recover bacteria, 250 µl SOC medium was added and mixture was incubated under 220 rpm shaking for 1 h at 37°C. Afterwards, the mixture was plated on LB-agar plates, containing the plasmid-corresponding antibiotic to enable selection of transformed clones and were incubated overnight at 37°C. Grown colonies were picked and used for inoculation of 5 ml LB medium with the appropriate antibiotic to ensure selection pressure. Shaking at 220 rpm, the liquid culture were left overnight at 37°C.

4.3.5. RNA interference

Originally in plants identified (Hamilton and Baulcombe 1999), relies this method on the cleavage of dsRNA into small (20-30 nucleotides) RNAs by the RNAse III-like enzyme dicer. These molecules are referred to as small interfering RNA (siRNA) (Table 4.20), from which the sense strand gets incorporated into the RNA induced silencing complex (RISC), leading to degradation of host mRNA complementary to the load.

Table 4.20 | siRNA used within this thesis

Target gene	Cat. No. (Quiagen)
AllStars	1027280
HK1	SI02225195
HK2	(a) SI00287336
	(b) SI00287329
	(c) SI03083479
	(d) SI00004074
PKM2	SI00288022

The reagent HiPerFect is a combination of cationic and neutral lipids which enable siRNA uptake and subsequent release inside cells. It was gently mixed with siRNA and cold medium (Table 4.21). The mixture was incubated for 30-40 min at RT, during which AGS were splitted as usual (see “Cultivation”), counted using a hemocytometer and a cell dilution was prepared, yielding in 2 x 10^4 cell in 400 µl. Following incubation of the transfection mix, it was gently mixed and pipetted into a fresh 24 well plate. From the cell suspension 400 µl was added to each well and cells were incubated for 72 h under normal cultivation condition. Of note, gene silencing is eventually stopped when incubation longer, as RNAi is lost in lineage.

Table 4.21 | siRNA transfection for 24 well plate

Compound	Volume [µl]
siRNA (final: 20 nM)	1
Volume of HiPerFect Reagent (Qiagen)	6
cold, serum-free cell culture medium	93

4.3.6. CRISPR/Cas9 knockout

Bacteria and Archaea rely on defense mechanism to withstand viral or nucleic acid invasion. This is provided by clustered regularly interspaced short palindromic repeats (CRISPR) which target nucleic acid sequence-specific, enabling inheritable DNA-encoded immunity (Horvath and Barrangou 2010). In more than 40% of bacteria and 90% of archaea, CRISPR sequences are found. CRISPR-associated (Cas) systems use short CRISPR RNA (crRNA) and transactivating CRISPR RNA (tracrRNA) to direct Cas nuclease to a target sequence in the genome. Immediately downstream of the crRNA binding site, Cas-specific protospacer adjacent motif (PAM) is needed to initiate nuclease activity, producing a double-strand break 3-5 nucleotides upstream. With non-homologous end-joining or homologous recombination, the DSB is repaired, which often introduces indels or frameshift mutations (Reviewed in (Adli 2018)).

In this thesis, the one-vector system plentiCRISPRv2 from addgene was used, which encodes *S.pyogenes* CRISPR-Cas9 and a custom-made chimeric sgRNA. It is composed of crRNA which hybridizes with complementary base pairing of the target DNA and the tracrRNA sequence.

Design of single-guide RNA

To design the crRNA needed for HK2 specific KO, the online tool Chop Chop was used. Alignment algorithms are based on large scale studies, used to calculate off-target potential (Labun, Montague et al. 2016). Nevertheless, knockout efficiency is difficult to predict, thus three different target sites were chosen (Table 4.22).

Table 4.22 | Target sites predicted by ChopChop

Gene	Accession no	Construct	target sequence (5'-3')	Exon
HK2	NM_000189	I. II III.	CTTGATGCGCTGCAGCACGGCGG GTGCCTGCCAAAACAAGGGACGG TGCAGAAGGTAAGTCAGCGCG	9 7 1

(designed on the 2017-09-13).

sgRNA Cloning

To clone the designed crRNA, an oligonucleotide (oligo) for the respective forward and reverse sequence was synthesized. The previously selected target sequences without PAM served as input and *Bsm*BI overhangs were included to enable target vector ligation. The sgRNA scaffold of plentiCRISPRv2 is flanked by *Bsm*BI restriction sites (Table 4.23, underlined) to enable digestion and subsequent cloning of the annealed oligos.

Table 4.23 | Cloning oligos for plentiCRISPRv2

Construct	Target sequence (5'-3')	PAM	Fw Oligo (5'-3')	Rv Oligo (5'-3')
I	CTTGATGCGCTG CAGCACGG	CGG	CACCGCTTGATGCGCT GCAGCACGG	AAACCCGTGCTGCAG CGCATCAAGC
II	GTGCCTGCCAA AACAAGGGA	CGG	CACCGGTGCCTGCCA AAACAAGGGA	AAACTCCCTTGTTTT GGCAGGCACC
III	TGCAGAAGGTAA GTCAGCGC	GGG	CACCGTGCAGAAGGT AAGTCAGCGC	AAACGCGCTGACTTA CCTTCTGCAC

Sterile, nuclease-free water was used to reconstitute lyophilized oligos for a final concentration of 100 µM. To enable ligation int the target vector, resuspended oligos were phosphorylated and subsequently annealed (Table 4.24). The reaction mix was incubated at 37°C for 30 min. Afterwards, to inactivate the ligase, temperature was increased to 95°C for 5 min and then ramped down to 25°C at 5°C per minute.

Table 4.24 | Master mix for annealing and phosphorylation of oligos.

Component	Stock concentration	Volume	Final concentration
Fw oligo	100 µM	1 µl	10 µM
Rv oligo	100 µM	1 µl	10 µM
T4 Ligation Buffer	10x	1 µl	1x
ddH_2O	-	6.5 µl	-
T4 Polynucleotide Kinase	10 000 units/ml	0.5 µl	0.5 U/µl

To linearize and enable cloning, the plentiCRISPRv2 was digested and dephosphorylated. The reaction (Table 4.25) was carried out at 37°C for 30 and purified from agarose gel afterwards.

Table 4.25 | Master mix for plentiCRISPRv2 digestion and dephosphorylation.

Component	Stock concentration	Volume	Final concentration
lentiCRISPRv2 plasmid	820 ng/µl	6 µl	5 µg
*BsmB*I		3 µl	
FastDigest Buffer	10 x	6 µl	1x
FastAP		3 µl	
DTT	100 mM	0.6 µl	1 mM
ddH_2O		38 µl	

The previously phosphorylated and annealed oligos were cloned into the digested and dephosphorylated plasmid (Table 4.26). Quick Ligase was used to facilitate phosphodiester binding between 5'-phosphate and 3'-hydroxyl termini.

Table 4.26 | Master mix for ligation of oligo complex into p lentiCRISPRv2

Component	Stock concentration	Volume	Final concentration
*BsmB*I digested lentiCRISPRv2	90 ng/µl	0,6 µl	50 ng
Annealed oligos	1:200 diluted	1 µl	
Quick Ligase Buffer	2x	5 µl	1x
Quick ligase		1 µl	
ddH_2O		3,4 µl	

The reaction was carried out at RT for 4h and followed by overnight incubation at 8°C. The resulting mixture was stored at -20°C and subsequently directly used for transformation into Stbl3 bacteria.

4.3.7. RNA Microarray

Two biological replicates were used to perform microarray experiments according to manufacturers' instructions, by dual-color hybridization on a custom whole genome human 8x60K G3 Agilent array platform (Design ID 048908). Agilent Feature Extraction software was used to acquire probe intensities. RNA isolation, quality assessment using the Bioanalyzer, probe labeling, hybridization, final scanning and analysis were performed by Dr. Hans Mollenkopf (MPIIB core facility Microarray).

To obtain enough cells, each experimental condition was performed in technical triplicates of a 6-well plate. Following the experiment, medium was aspired and cells from each condition were dissolved and pooled in 1 ml of TRIzol. Following RNA extraction, quantity was assessed spectrophotometrically (NanoDrop1000) and quality was asses using the RNA Nano 6000 microfluidics kit for the Agilent 2100 Bioanalyzer (Agilent Technologies). RNA labeling was performed according to the Agilent Two-color Low Input Quick Amp Labeling Kit protocol. Labeled probes were hybridized to the assay and scanning was performed using the Agilent high-resolution laser microarray scanner. Final analysis was performed using the BioConductor package LIMMA (Ritchie, Phipson et al. 2015).

4.4. Protein techniques

4.4.1. Western Blotting

Cell Lysis

To detect intracellular proteins, cellular integrity has to be disrupted using detergents. Supernatant was discarded and cells were lysed and proteins denaturated with 2x Laemmli buffer (100 µl /24 well plate well) (Table 4.27). The buffer contains the cell-disrupting anionic detergent sodium dodecyl sulfate (SDS). Cells suspension was heated in a thermo shaker with 500 rpm for 10 min at 96°C and stored at -20°C.

Table 4.27 | Laemmli Buffer

Component	Concentration
Tris (adjusted to pH 6.8 with HCl)	120 mM
Glycerol	20 %
SDS (Stock 10 % (w/v))	4 %
Bromphenol blue	0.02 %
β-mercaptoethanol:	5 %

used diluted to 2x with Milli-Q H_2O

SDS-PAGE

SDS-PAGE (Sodium dodecyl sulfate Polyacrylamide gel electrophoresis) enables the electrophoretic separation of proteins according to their mass. The matrix is based on a discontinuous polyacrylamide gel and the detergent SDS denatures proteins and charges the protein surface negatively, leading to migration according to size and not protein charge.

To separate proteins, the Bio-Rad Mini-PROTEAN system was used with Tris-glycin running buffer (Table 4.28) and 4–15 % Mini-PROTEAN® TGX™ Precast protein gels. As protein marker, the prestained PageRuler™ was used. To run the electrophoresis, a constant voltage of 120 V was applied for 90 – 120 min.

Table 4.28 | SDS PAGE running Buffer

Component	Concentration
Tris	248 mM
Glycin	1.52 M
SDS (Stock 10 % (w/v))	1 %
add ddH_2O pH 8.3	

Protein transfer

To enable protein transfer from the polyacrylamide gel to a polyvinylidene difluoride (PVDF) membrane, proteins are mobilized via electric current. Membrane was activated in methanol for 5 min and subsequently equilibrated in transfer buffer (Table 4.29) for 5 min, as was the SDS-PAGE gel, four blotting paper and two sponges. The following pile was built from anode to cathode: sponge-two blotting paper – membrane – gel – two blotting paper – sponge and placed into the wet blot tank. The tank was filled with transfer buffer, along with a cooling pack and the

transfer was performed at 250 mA for 2,5 hours at 4°C and continuous stirring to enable homogenous cooling.

Table 4.29 | Protein transfer buffer

Component	Concentration
For 10x:	
Tris	250 mM
Glycin	1.90 M
ad ddH_2O	
For 1x:	dilute 1:10
add Methanol	20 %

Protein staining

After transfer, the membrane was washed for 5 min with washing buffer (TBS-T) (Table 4.30) on a rolling platform.

Table 4.30 | Western blot washing & blocking buffers

Component	Concentration
10 x TBS:	
Tris	20 mM
NaCl	140 mM
add ddH_2O, adjust pH 7.5	
For TBS-T (washing buffer):	dilute 1:10
add Tween-20	0.1 %
For blocking buffer:	
TBS-T	
add milk powder / BSA	5 % / 3 %

The proteins are bound to the PVDF membrane and can be detected via antibodies (Table 4.31). To avoid unspecific protein binding based on hydrophobic and charge-mediated interactions with of the PVDF membrane, the membrane was incubated with a blocking buffer for 1 h at RT on a rolling platform. Following blocking, the membrane was washed five times with TBS-T for 5 min at RT on a

rolling platform. Afterwards, the Membrane is incubated with the antibody specific for the protein of interest (primary antibody), diluted in blocking buffer containing BSA, overnight at 4°C on a rolling platform. Following incubation, the membrane is washed with TBS-T three times for 15 min on a rolling platform. Subsequently, the membrane is incubated with a horse radish peroxidase (HRP)-coupled secondary antibody, for 2 h at RT. Following incubation, the membrane was washed three times with TBS-T for 10 min at RT on a rolling platform.

Table 4.31 | Antibodies used for western blot analysis.

Target	Reactivity	Dilution	Dye	Host	Supplier	Catalog#
Beta actin	Human	1:3 000	None	Mouse	Sigma Aldrich	A5441
CagA	*H. pylori*	1:1 000	None	Rabbit	Santa Cruz	Sc-25766
p-Tyr 99	None	1:1000	None	Mouse	Santa Cruz	Sc-7020
PD-L1	Human	1:1 000	None	Rabbit	abcam	ab228415
IgG (H+L)	Mouse	1:3 000	HRP	Sheep	GE Healthcare	NA931
IgG (H+L)	Rabbit	1:3 000	HRP	Donkey	GE Healthcare	NA934

Following washing, equal volumes of each reagent from the ECL Western Blotting Substrate kit were mixed and 1 ml of the mixture was added to the membrane for 1 min at RT enabling detection of HRP enzyme activity. Then, the membrane was placed in a photo cassette and protein visualization took place in a dark room. It is based on the ingredient of the reagents: the chemiluminescent reagent luminol and H_2O_2. In the presence of the latter, HRP oxidizes the luminol, a process known to emit light, which can be detected using a photosensitive film, Hyperfilm (Amersham Biosciences), and a developer machine.

4.4.2. Immunofluorescence Staining and Microscopy

Cell seeding

For immunofluorescent staining, a sterile coverslips was placed in a well of the 24 well-plate prior to cell passaging. Cells were treated and seeded as usual (for cell lines, see subchapter “Cultivation” and for primary cells, see subchapter “2D cultivation”).

Sample preparation

Liquid was discarded and cells were washed with warm PBS. Cells were fixed with 1 ml of 4 % formaldehyde in PBS for 15 min at RT. Fixative was aspired and cells were rinsed with warm PBS for 5 min each.

Methanol Permeabilization

To permeabilize the cell membrane and ensure better staining of proteins, cells were incubated with ice-cold 100% methanol for 10 min at -20°C and rinsed three times with PBS afterwards.

Immunostaining

The process of immunofluorescence visualizes proteins by labeling them with a specific primary antibody and a secondary antibody (Table 4.32), which is coupled to a fluorescence dye and binds the primary antibody. By illumination of a wavelength specific for the fluorophore, light is emitted and can be assessed microscopically.

Table 4.32 | Antibodies used for immunofluorescence microscopy.

Target	Reactivity	Dilution	Dye	Host	Supplier
PD-L1	human	1:1 000	none	rabbit	abcam
IgG (H+L)	rabbit	1: 500	Cy2	donkey	Dianova

The staining was carried out at RT, unless noted otherwise. The specimen was incubated in blocking buffer (Table 4.33) for 60 min. While blocking, primary antibodies are diluted as indicated above using the antibody dilution buffer (Table 4.33). Then, blocking solution is aspired and antibody dilutions are applied and incubated overnight at 4°C. Afterwards, specimen are washed three times with PBS for 5 min each and secondary antibodies are diluted as in antibody dilution buffer. Incubation is performed for 2h using a humid light-tight box to prevent drying and fluorochrome fading. Cells were rinsed three times with PBS for 5 min each. Excess liquid was removed and mowiol was used as mounting medium to embed up to three coverslips onto one glass slide. The glass slide was left for drying for at least 1 h under light-tight conditions. Finally, the coverslip was sealed with nail polish and stored light-protected at 4°C.

Table 4.33 | Immunofluorescence blocking & antibody dilution buffers

Component	Concentration
1x PBS	
Triton™ X-100	0.3 %
For blocking buffer	
normal serum (e.g. donkey serum)	5 %
For antibody dilution buffer:	
BSA	1 %

Image acquisition

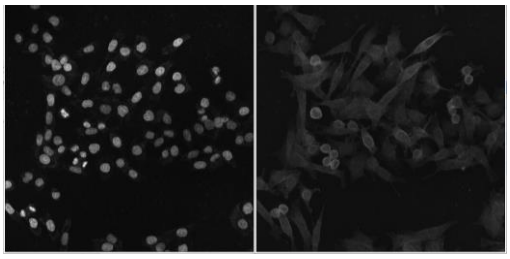
Following staining, images were acquired at the Leica TCS SP-8 confocal microscope (Leica Microsystems GmbH) with 10x, 20x or 40x oil objective using the respective Leica software (Leica Microsystems GmbH).

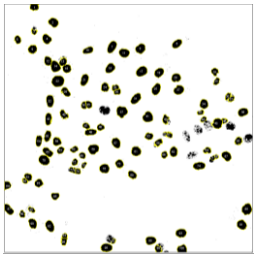
Image processing

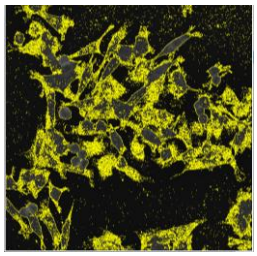
For image processing, the software Fiji ImageJ (open source) was used. In order to quantify protein expression of PD-L1, a two-step process using ImageJ was followed (Table 4.34).

First, from the image, which was opened with split channels, the channel containing the nucleic staining was selected and inverted to allow counting the number of nuclei. The optimal particle size was initially determined and kept throughout the analysis. In the second step, the channel containing the staining for PD-L1 protein expression was selected. A scaffold surrounding the cells was created and the overall intensity within the scaffold was determined. Of note, every image had the same number of z-stacks to enable comparison. Finally, overall intensity was divided by the number of nuclei. Uninfected condition was set to 1 and infection conditions were normalized to it.

Table 4.34 | Image processing using ImageJ

	ImageJ Command	Result example
Original		

Step 1	run("Z Project...", "projection=[Max Intensity]");
	setOption("BlackBackground", false);
	run("Make Binary");
	run("Fill Holes");
	run("Watershed");
	run("Analyze Particles...", "size=200-Infinity pixel circularity=0.20-1.00 display clear summarize add");
		

Step 2	run("Z Project...", "projection=[Max Intensity]");
	setOption("BlackBackground", false);
	run("Make Binary");
	roiManager("deselect");
	roiManager("delete");
	run("Create Selection");
	roiManager("Add");
		

4.4.3. Preparation of lentivirus

Lentiviruses belong to the group of retroviruses, able to infect a range of cell types and organism. Its genome consists of RNA, which is, upon infection of the host, converted into DNA and integrated into DNA of the host, enabling its replication. Given that lentiviruses are able to infect dividing as well as non-dividing cells, they have a high transduction efficiency and are widely used. To increase the usage of viruses, only the plasmid containing the desired insert sequence is transported into the host and subsequent incorporation into the host genome. Viral compartments are encoded by additional plasmids, such as packaging vector or envelope vector to not interfere with the mammalian genome. For the generation of stable CRISPR/Cas9 knockout cell lines, a lentiviral transfer plasmid (pLentiCRISPRv2), an envelope vector (pMD2.G) and a packaging vector (psPAX2) were used to transiently transfect into the packaging cell line HEK 293T (50% confluency, seeded in a 10 cm^2 dish). A mixture of 15.6 µlFuGENE6 and 192.4 µl OptiMEM was

prepared and incubated for 5 min at RT. The lentiviruses were diluted as indicated below (Table 4.35), mixed with the FuGENE mixture and incubated for 30 min at RT.

Table 4.35 | Dilution of lentiviral plasmids for transfection

Component	Concentration
lentiviral transfer plasmid (lentiCRISPRv2 construct)	2.6 µg
packaging vector (psPAX2)	1.95 µg
envelope vector (pMD2.G)	0.65 µg
OptiMEM add 52 µl	

The final mixture was gently added to HEK 293T cells, incubated at 37°C and medium was replaced 12-15 h later. Medium of the cells contains the viruses and was collected 48 h later, following filtration with a 0.45 µl syringe and was directly used for mammalian cell transduction.

4.4.4. Transduction of mammalian with lentiviruses

To introduce exogenous DNA into mammalian genomes, viruses can be used. Lentiviral transduction was used to generate a stable CRISPR/Cas9 knockout AGS cell line. AGS were seeded in 10 cm^2 dish and medium was replaced with lentivirus-containing medium (see “Preparation of lentivirus”) when cells reached a confluency of 50 %. Polybrene (5 µl) was added to enhance viral adsorption on AGS cell membranes. Cells were incubated for 24 h and subsequently, 0.4 µg/ml puromycin was added to select for cells, containing the plasmid. Antibiotic selection was continued for 10 days with fresh medium every day.

4.4.5. Generation of CRISPR/CAS9 KO single cell lines

Cells, obtained from transduction contain a polyclonal cell population. Limiting dilution was used to obtain single clones for a monoclonal cell line. Cells were splitted, counted and diluted to a concentration of 5 cells/500µl, from which 100 µl were added into each well of a 96-well plate. After 7-10 days of cultivation, wells were screened for single cell colonies which were expanded and screened for target gene deletion.

4.5. Metabolite techniques

4.5.1. Carbohydrate experiments

Protocols for experiments, performed by Dr. Robert Hurwitz, which include synthesis of HBP and ADP heptose, derivatization of heptoses, MALDI TOF MS and UPLC can be found in (Pfannkuch, Hurwitz et al. 2019).

Lipofectamine transfections

Lipofection was applied actively transport the sugars ADP heptose, HBP and LPS (LPS-EB Ultrapure, InvivoGen, Cat.# tlrl-3pelps) into the periplasm of the cells. The technique is based on the reagent that contains lipid subunits which, in aqueous environment, form liposomes able to entrap sugars. After addition to the cell, the lipids merge with the cell membrane, releasing the cargo into the cytoplasm.

On the day prior to transfection, AGS were seeded in a 24 well plate to gain 2 x 10^5 cells per well on the next day. Prior to the experiment, cells were starved in serum-free cell culture medium for 2-3 h at 37°C. All compounds (Table 4.36) were mixed and preincubated for 20 min at RT. Serum-free medium was added to the transfection mixture and gently pipetted. Cell medium was aspirated. Then, cells were covered with transfection-medium mix and incubated for 3 h at 37°C and 5 % CO_2.

Table 4.36 | Lipofectamine Transfection

Component				Supplier
sugar	1 µl			
Opti-MEM	7.5 µl			Thermo Fisher Scientific
ATP (20mM)	6.25 µl			Roche, Basel, Switzerland
lipofectamine 2000	1.25 µl			Thermo Fisher Scientific

Depending on the desired analysis, cells were either lysed with subsequent luciferase assay, according to the manufacturers protocol (Promega) or cells were lysed and a qRT-PCR was performed

4.5.2. Pyruvate Measurement

Method was performed by Dr. Robert Hurwitz.

Table 4.37 | Pyruvate measurement

Component	Supplier
4-(N,N-Dimethylsulfamoyl)-7-piperazino-benzofurazan (DBD-PZ)	Sigma-Aldrich
Triphenylphosphine	Sigma-Aldrich
2,2′-dipyridyl disulfide	Sigma-Aldrich
1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride	Sigma-Aldrich
4-N,N-dimethylaminopyridine	Sigma-Aldrich

Pretreatment procedure

Samples were extracted by the method of Bligh and Dyer (Bligh and Dyer 1959). Briefly, the aqueous sample solution was mixed with two volumes of chloroform/methanol in 1:1 ration. After mixing and centrifugation, the aqueous phase was dried in a SpeedVac concentrator and dissolved in acetonitrile.

Precolumn derivatization

20 µl of the sample solution and 20 µl of a 0.1 mM methylsuccinic acid solution, which was used as internal standard, were added to a solution of 50 µl of 0.1 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, 50 µl of 0.1 M 4-N,N-dimethylaminopyridine and 10 µl of acetonitrile, in a light shielding tube. After adding 50 µl of a 40 mM DBD-PZ solution, the reaction mixture was heated at 60°C for 120 min. A 200 µl aliquot of 30% acetonitrile, containing 0.1% trifluoroacetic acid, was added to the mixture to stop the reaction. 50 µl aliquots were applied to sulfonated poly(styrenedivinylbenzene) polymer (SDB-RPS, Empore™) cartridge equilibrated 0.1% trifluoroacetic acid. Derivatized samples were eluted with 1 ml of acetonitrile and dried by speedVac-evaporation. The samples were dissolved in 100µl of acetonitrile–0.1% trifluoroacetic acid (1:1, v/v). 1-5 µl were injected onto a reversed phase UPLC-Column (ACQUITY UPLC HSS T3 Column, 1.8 µm, 2.1 mm X 100 mm).

UPLC Conditions (Waters H-Class)

Linear gradient from 30% acetonitril/ 0.1% TFA to 100 acetoniril/0.1%TFA in 5 min at a flow rate of 0.5ml/min. Column temperature: 50°C.

Detection with Waters Fluorescence Detector: excitation at 450nm, emission at 560nm.

4.5.3. Cell viability assay

The colorimetric assay is based on reduction of the yellow reagent 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) (Sigma-Aldrich) to the purple formazan. Thereby, activity of NAD(P)H-dependent oxidoreductase enzymes drives the reduction process, providing direct connection to metabolic activity, which reflects living, active cells. The reduction degree is measured spectrophotometrically using absorbance between 500-600 for the formazan product and a reference wavelength has to be assessed with absorbance of more than 650.

To the cells, containing media, 10 µl of 5 mg/ml of MTT was given and incubated for 2 h under cell culture conditions. Then, the plate was centrifuged (10 min, 2 000 rpm, RT) to collect the insoluble formazan crystals. The media is discarded and the crystals are dissolved using 200 µl of 40 mM acidified isopropanol for 15 min 37°C for 15 min under constant gentle shaking. Finally, a spectrophotometer(infinite M200 TECAN) was used, assessing formazan absorbance at 570 nm.

4.6. Statistical analysis

Graphs were prepared using Prism version 7 software. Data are presented as mean ±SEM (standard error of the mean), calculations for p-values was performed using a two-sided unpaired Student's t test. Data was considered as significant for p-value<0.05.

Chapter

5. Results

PD-L1 induction is a multifactorial event in *H. pylori* infection

The PD-1:PD-L1 signaling axis is essential for a functioning immunity, being involved in a range of processes, like peripheral tolerance, lymphocyte development and inflammation. Particularly in infection, the pathway regulates the balance between antimicrobial defense and inflammation-related tissue damage. In line with this, infection with the gastric pathogen *H. pylori* was shown to induce the ligand PD-L1 (Wu, Lin et al. 2010, Lina, Shatha et al. 2014). To understand the dynamics of the underlying activating mechanism, the gastric human cell line AGS was used and infected with wildtype (wt) *H. pylori* over a course of 48-hour (Figure 5.1). Changes of PD-L1 induction were measured via qRT-PCR after 3 h, 6 h, 12 h, 24 h, 48 h in respect to its expression at the beginning (0 h). It can be observed, that infection leads to immediate upregulation after only 3 h of infection and decreases afterwards. Another PD-L1 expression peak can be observed later, after 24 h.

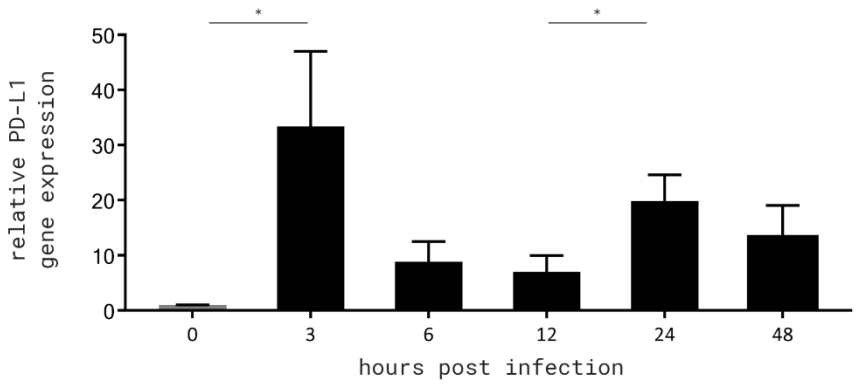


Figure 5.1 | During a 48h time course of *H. pylori* infection PD-L1 induction occurs in two phases
Analysis of qRT-PCR for three independent biological replicates of human gastric cancer cells (AGS), infected with *H. pylori*, MOI 50. Infection was stopped after 3 h, 6 h, 12 h, 24 h, 48 h. Relative PD-L1 mRNA expression was assessed via qRT-PCR in respect to gene expression at timepoint 0. Statistical analysis was performed using Student´s *t*-test (*, $p \leq 0.05$).

The large time span between the two induction phases points towards two different activation mechanism which will be studied in further detail below. The time around

the first peak will be following termed **acute response** and the later occurring induction will be referred to as **late response**.

5.1. Acute *H. pylori* infection response

The gastric bacterium is well-adapted to co-exist in the human stomach for decades by evading immune recognition through various strategies, including niche formation and PAMP modification. However, early infection are marked by elevated levels of inflammation in the epithelial lining, indicating towards innate immune mechanisms that are able to recognize bacterial compounds in order to stimulate this reaction.

5.1.1. NF-κB activation is crucial for PD-L1 induction

Infection with *H. pylori* led to PD-L1 induction at the very first measured time point, after 3 h. To determine the initial induction, another time course, covering the time around the acute response was performed (Figure 5.2 [A]). It showed, that induction onset has already occurred at the earliest measured time (1.5 h) and proceeds to increase with a peak around 3 h post infection, after which it declines. One of the first signaling cascades, found to be triggered in *in vitro H. pylori* infection, is activation of the NF-κB pathway (Belogolova, Bauer et al. 2013). This leads to nuclear translocation of the activated complex within 15 to 30 min post infection, binding to κB DNA regions of target genes, like the pro-inflammatory chemokine IL-8. To estimate the correlation between NF-κB signaling and PD-L1 induction, regulation of IL-8 was traced over the course of 6 (Figure 5.2 [B]). Upregulation was observed 1.5 h post infection with induction peak at 3 h, revealing a similar induction pattern between the chemokine and the immune regulator.

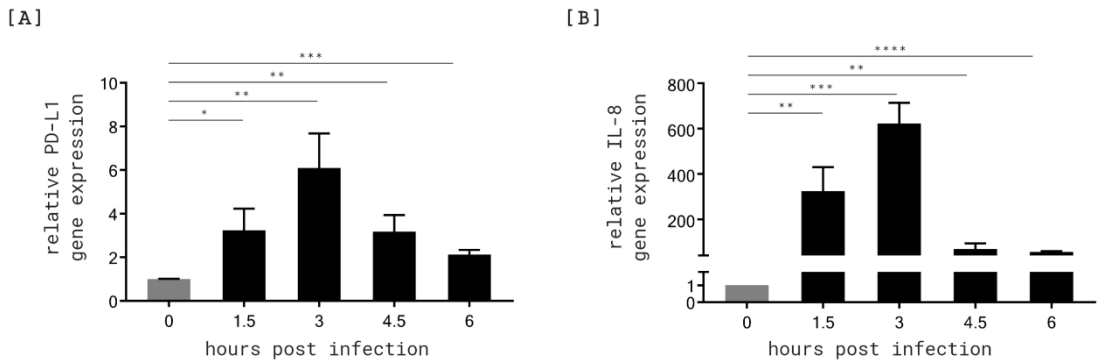


Figure 5.2 | PD-L1 and IL-8 share gene expression pattern
qRT-PCR of AGS, infected with *H. pylori* at a MOI of 50. Infection was stopped after 1.5 h, 3 h, 4.5 h, 6 h. Relative mRNA expression was assessed via qRT-PCR in respect to gene expression at timepoint 0. Three independent replicates were performed. Statistical analysis was performed using Student´s *t*-test (*, $p \leq 0.05$). [A] Relative PD-L1 gene expression. [B] Relative IL-8 gene expression.

It was shown for *H. pylori*, that early activation of NF-κB is triggered through an intermediate of the ADP heptose pathway, which is translocated into the host cell via a secretion system. Infection was performed with ΔrfaE bacteria as well as strains deficient for proteins, forming the cagT4SS (Δ*cagPAI*). Both mutants displayed a significantly reduced upregulation for PD-L1 gene compared to wildtype *H. pylori* with more than 2 fold decrease (Figure 5.3 [A]). To confirm, that transcriptional upregulation leads to protein expression, AGS infected for 6 h with *H. pylori* wt and Δ*rfaE* were subjected to fluorescently labeled anti-PD-L1 antibodies and intensity was determined (Figure 5.3 [B] and [C]). PD-L1 intensity was calculated as average of PD-L1 intensity divided by number of nuclei, leading to the observation, that protein intensity is significantly enhanced upon wildtype infection. The ligand exerts its function as transmembrane protein upon binding with the receptor PD-1, giving a crucial role to its cellular location (Freeman, Long et al. 2000). Expression is mainly observed at the cell borders, indicating a membranous position, which is needed for interaction with the receptor. Not infected cells also show a low level basal PD-L1 expression.

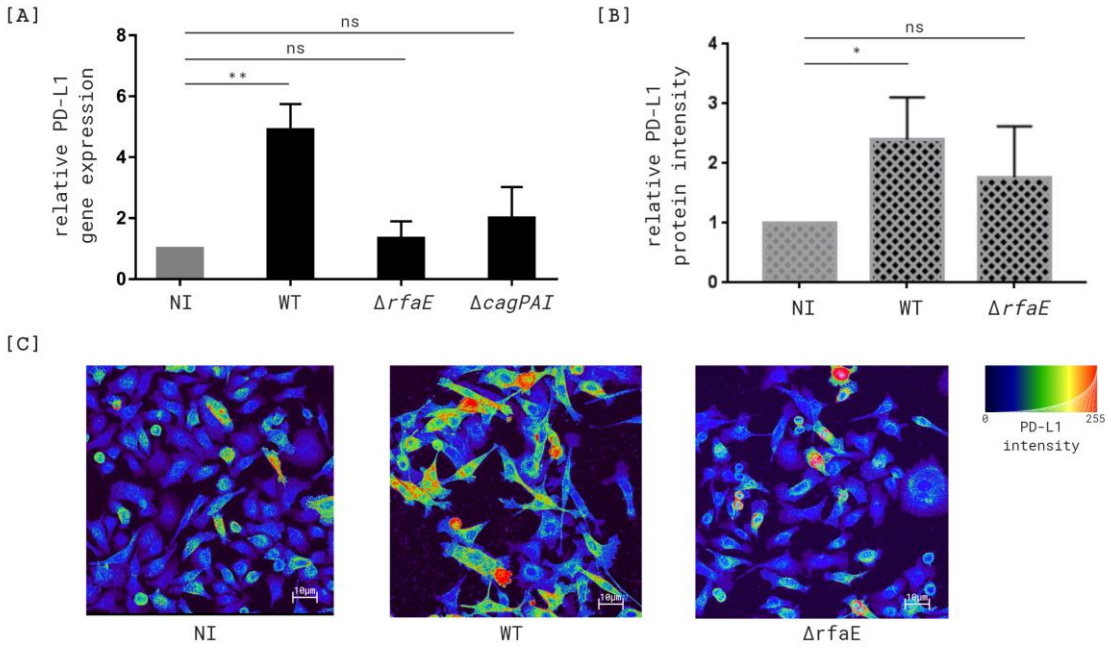


Figure 5.3 | T4SS cagPAI and ADP heptose synthesis pathway are essential for acute PD-L1 regulation

Statistical analysis was performed using Student´s *t*-test (*, $p \leq 0.05$). [A] AGS infected with *H. pylori*, MOI 50 wildtype (wt), Δ*cagPAI*, Δ*rfaE* or left uninfected for 3h. Relative PD-L1 mRNA expression was assessed via qRT-PCR in respect to gene expression of uninfected sample. [B-C] AGS infected with *H. pylori*, MOI 50 wt, Δ*PAI*, Δ*rfaE* for 6 h. Upon infection, cells were fixed and subjected to anti-PD-L1 antibody (red) and DAPI (blue) and subsequently analyzed by confocal microscopy. Experiment was performed in three independent replicates. [B] Quantification of PD-L1 protein expression in IF: Overall PD-L1 expression intensity of section was divided by number of nuclei. For each condition, two sections were analyzed. Statistical analysis was performed using Student´s *t*-test (*, $p \leq 0.05$). [C] Representative images of immunostainings.

5.1.2. HBP is not the NF-κB activating compound in H. pylori lysates

Most results from the following two subchapter are published in the paper "ADP Heptose, a Novel Pathogen-Associated Molecular Pattern Identified in *Helicobacter pylori*" (Pfannkuch, Hurwitz et al. 2019).

In a range of Gram-negative bacteria (*Shigella flexneri*, *Salmonella typhimurium*, *Neisseria meningitidis* and *H. pylori)* it has been reported that the LPS synthesis intermediate HBP induces NF-κB (Milivojevic, Dangeard et al. 2017, Zimmermann, Pfannkuch et al. 2017). This sugar, which was first linked to inflammation in *Neisseria* infection, is produced within the multi-step biosynthesis pathway of ADP heptose, with the heptose residue being an integral part of the LPS inner core

region (Gaudet, Sintsova et al. 2015). Knowledge about the sugar arose from experiments with bacterial mutants, deficient for enzymes involved in ADP heptose production. Bacterial lysates and *in vitro* synthesized HBP were tested for NF-κB inducing capacity. However, no study provided evidence, that bacterial lysates contain HBP in sufficient amounts to induce a signaling cascade. To tackle this challenge, an unbiased approach was used, in which the compound of bacterial lysates, responsible for NF-κB activation was characterized:

I. Extraction of NF-κB stimulating fraction from bacterial lysates

Solid phase extractions/SPEs of *H. pylori* lysates were separated by reverse phase/RP – ultra performance liquid chromatography/UPLC and collected. Resulting fractions were tested for their capacity to activate NF-κB. As read-out, luminescence of AGS, stably expressing a construct, containing NF-κB response elements which drive transcription of a luciferase-encoding gene were used. Bacterial lysate fractions were introduced into cells by lipofection, as it was shown that active transport into the host is necessary (Zimmermann, Pfannkuch et al. 2017). Clear determination of the activating bacterial-derived fraction was possible, as shown in Figure 5.4.

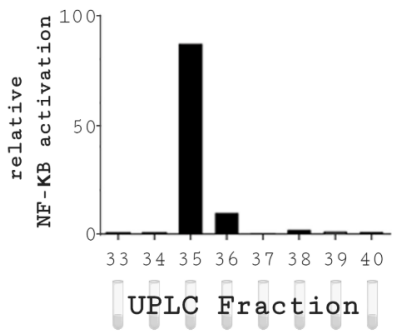


Figure 5.4 | NF-κB stimulating, UPLC separated, *H. pylori* fraction
H. pylori SPE were separated via RP-UPLC, collected fractions were introduced into AGS NF-κB luciferase reporter cells by lipofection. After 3 h, activation was measured by luminescence for a given fraction, relative to the signal of untreated cells. Experiment performed by Jan Traulsen.

II. HBP derivatization

To determine the amount of HBP in bacterial lysates, a chemically synthesized version was characterized by mass spectrometry as reference, revealing a mass of *m/z* 369 $[M-H]^-$ to be specific for this sugar. Detection of HBP from bacterial SPEs was not possible, as no specific mass was found. A possible reason is the detection limitation for HBP with concentration ≥100 pmol. In mass-spectrometric analysis, a

frequently used strategy to increase low sensitivity is by derivatization of samples (Qi, Liu et al. 2014). The compound 3-Amino-9-ethylcarbazole/AEC was used (Figure 5.5 [A]), as derivatization occurs rapidly under mild conditions and the resulting introduction of fluorophores enhances UV separation as well as reversed-phase LC retention. In monosaccharides, derivatization with AEC via reductive amination leads to derivatized sugars with lower detection limits (Zhang, Huang et al. 2007). Furthermore, AEC was shown to provide good results for selective determination of phosphorylated reduced sugars in mixed samples (Han, Tschernutter et al. 2013). To chemically reduce the sugar HBP and thereby freeing an aldehyde group, needed for subsequent AEC derivatization, two hydrolytic approaches were applied; (1) Trifluoroacetic acid/TFA was used, as the glycosidic linkage, which is present between the sugar moiety on the C1 and the phosphate, is sensitive to acid-catalyzed hydrolysis, (2) Calf intestinal alkaline phosphatase/CIP reaction led to removal of the two phosphate groups. Both processes led to generation of an aldehyde group which was then subjected to reductive amination. The finally formed amines were analyzed by mass spectrometry, revealing masses of *m/z* 485 $[M+H]^+$ for TFA-treated and *m/z* 405 $[M+H]^+$ for CIP-treated HBP.

III. Characterization of derivatizes HBP and bacterial fraction

To characterize the resulting HBP molecules, MALDI-TOF tandem mass spectrometry (MSMS) was used. In this approach, molecules are ionized and separated by the first spectrometer according to their *m/z.* Ions of the previously identified mass (*m/z* 485 $[M+H]^+$and *m/z* 405 $[M+H]^+$), are selected (selected-ion recording or SIR) and fragmented. The second spectrometer separates the resulting fragments according to their *m/z*-ratio, leading to a precursor-specific fragmentation pattern. In spectra of both precursor molecules, a common fragment ion of *m/z* 223 $[M+H]^+$ was identified (Figure 5.5 [B]) and was subsequently used as marker, specific for derivatized HBP.

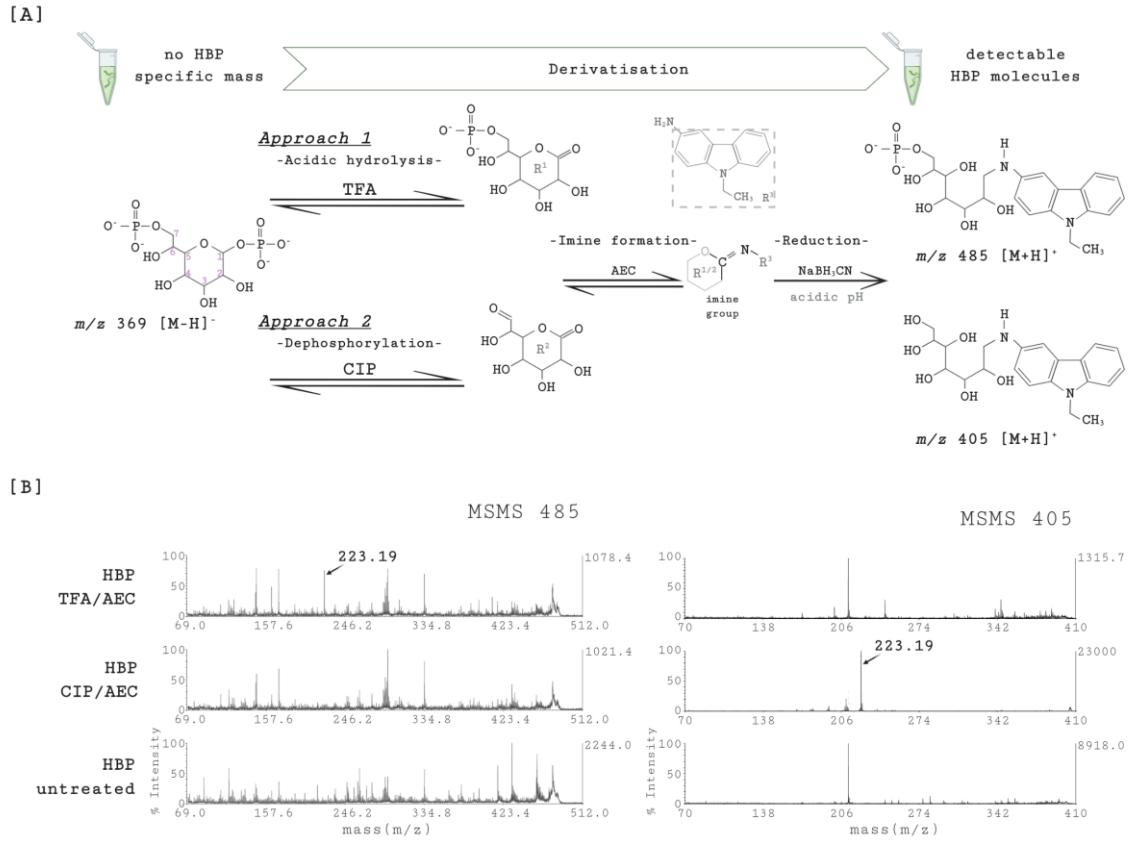


Figure 5.5 | Fragment ion *m/z* 223 $[M+H]^+$is specific for derivatized HBP

[A] Mass spectrometric analysis of H. pylori lysates SPEs detected no HBP. To increase detectable concentration, HBP was derivatized with AEC which binds to aldehyde groups. To reduce sugar, TFA and CIP were used, enabling subsequent derivatization and detection of HBP compounds with distinct m/z values. [B] Chemically synthesized HBP was pretreated with TFA or CIP to be derivatized with AEC, subjected to RP-UPLC and analyzed by MALDI-TOF MSMS with selected-ion of m/z 485 and m/z 405. Experiment performed by Dr. Robert Hurwitz.

Derivatization decreased detection limit of HBP to concentration ≥1 pmol, enabling detection in *H. pylori* lysates. NF-κB stimulating fraction was derivatized, separated and analyzed. Mass peaks were compared with mass peaks produced by chemically synthesized HBP as control (Table 5.1). SIR for *m/s* 485 $[M+H]^+$ led to a similar mass detection pattern for both hydrolysis approaches. SIR for *m/s* 405 $[M+H]^+$ on the other hand, revealed a distinct pattern for bacterial samples with only minor mass peaks after phosphatase treatment compared to HBP. Mass peaks following acidic treatment showed high masses in lysate fraction. To conclude, NF-κB-activating fraction from bacterial lysates displayed distinct fragmentation pattern compared to chemically synthesized HBP, indicating towards a lysate molecule,

different from HBP. Further proof was provided, as bacterial lysates containing 9 x 10^8 bacteria/ml, revealed HBP concentrations of approximately 0.1 µM which is too low to activate NF-κB (Zimmermann, Pfannkuch et al. 2017).

Table 5.1 | Fragmentation pattern of derivatized HBP and *H. pylori*

	Precursor	**Fragment**		
***m/z* [M+H]⁺**	**485**	**223**		
	HBP	***H. pylori*, NF-κB fraction**	**HBP**	***H. pylori*, NF-κB fraction**
TFA/AEC	75	38	783	88
CIP/AEC	0	0	88	0
untreated	0	0	0	0
	Precursor	**Fragment**		
m/z [M+H]⁺	**405**	**223**		
	HBP	***H. pylori*, NF-κB fraction**	**HBP**	***H. pylori*, NF-κB fraction**
TFA/AEC	0	1.230	0	5.665
CIP/AEC	12.850	9	30.959	137
untreated	0	0	0	0

IV Enzymatic digestion of bacterial fraction

For *H. pylori* infection, it was shown that ALPK1-driven NF-κB activation depends on the bifunctional enzyme RfaE of the ADP heptose synthesis pathway (Zimmermann, Pfannkuch et al. 2017). The enzyme catalysis the second step through phosphorylation activity, leading to production of HBP and in the fourth step, the adenylyltransferase activity generates ADP-D-glycero-D-manno-heptose (Figure 5.6 [A]). Molecules downstream of RfaE and different from HBP were suspected as the unknown compound in bacterial lysate, responsible for NF-κB signaling. This was supported as mass spectrometry analysis of the activating lysate fragment, identified a mass of *m/z* 618 $[M-H]^-$, clearly different from the HBP specific mass of *m/z* 369 $[M-H]^-$, which was only detectable after the sugar was added additionally (Figure 5.6 [B]). For both, lysate-derived and HBP- derived ions, masses were found in SIR for *m/s* 485 $[M+H]^+$ after TFA hydrolyzation (Table 5.1),

pointing towards a precursor molecule with an acid-labile glycosidic bond. In the synthesis pathway, downstream of RfaE, all four manno-heptoses contain such a linkage between the sugar moiety on the C1 of the heptose molecule and the phosphate (Figure 5.6 [A]). The rather minor mass peaks, detected with SIR for *m/s* 405 $[M+H]^+$ after CIP compared to HBP control, indicate the presence of partially resistant or unsusceptible phosphate groups. In the synthesis pathway, the sugar derived from HBP reaction is the compound D-glycero-D-heptose-1-P and contains a C1 bound phosphate group, which is similarly susceptible to phosphatase treatment. Therefore, an ADP heptose epimer was considered to be the unknown compound. Unique to these sugars among the molecules of the synthesis pathway is the presence of a phosphate diester, which is around 10^6 fold less susceptible towards phosphatase treatment than phosphate monoester (O'Brien and Herschlag 2001). To verify diester presence, *H. pylori* lysate SPEs were digested with phosphodiesterase (PDE) and phosphatase (Figure 5.6 [C]). Chromatograms in SIR mode for the previously identified mass *m/z* 618 showed complete hydrolyzation via PDE and only a minor effect of CIP treatment, providing further proof for ADP heptose.

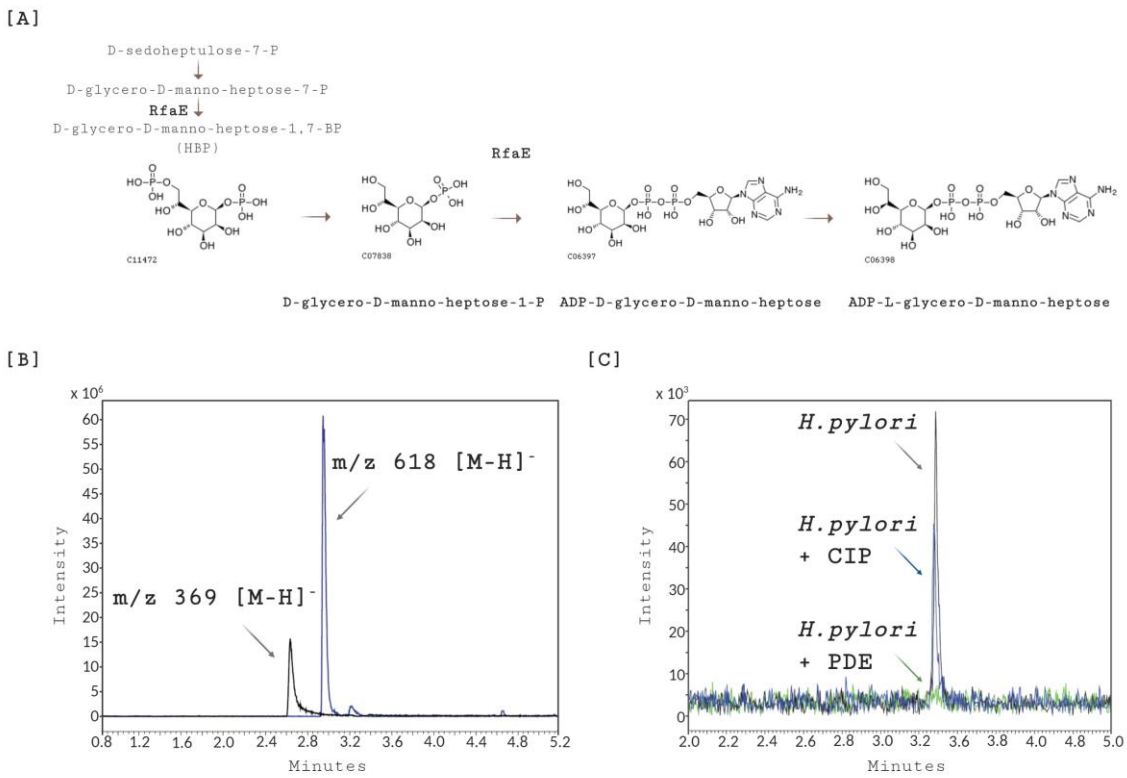


Figure 5.6 | Mass of m/z 618 [M-H]$^-$ and phosphatase-susceptibility of NF-κB activating compound

[A] Chemical structures of ADP heptose synthesis metabolites downstream of RfaE-driven reaction are illustrated. Structures were taken from KEGG database. Respective KEGG compound number is noted. [B] Chromatogram of NF-κB activating RP-UPLC fraction from *H. pylori* SPE (blue line, *m/z* 618), spiked with HBP (black line, *m/z* 369). [C] *H. pylori* SPE were left untreated (black line), digested with intestine alkaline phosphatase (CIP, blue line) or with phosphodiesterase (PDE, green line). Chromatograms are in SIR mode for *m/z* 618 [M-H]$^-$. Experiments performed by Dr. Robert Hurwitz.

5.1.3. ADP heptose is the NF-κB activating compound in H. pylori lysates

To identify ADP heptose as the unknown compound and to quantify it, MALDI-TOF MSMS was used. As reference, ADP heptose was synthesized *in vitro*. The two applied methods, chemical and enzymatical synthesis, yielded in moderate amounts of the expected β-from in L and D isomers. Chromatograms in SIR mode *m/z* 618 showed similar fragment ions for both sugars (Figure 5.7). Main fragment ions could be attributed to phosphate (*m/z* 97), adenine (*m/z* 134), pyrophosphate (*m/z* 159), ribose-phosphate (*m/z* 211), heptose-phosphate (*m/z* 289), AMP (*m/z* 346), and ADP (*m/z* 426), together with masses corresponding to the water-eliminated molecules. MALDI-TOF MSMS of *H. pylori* lysates showed ion intensities

for phosphate, adenine and AMP, which clearly identified ADP heptose as *m/z* 618 precursor in the bacterial lysate, in concentration of up to 1 µM.

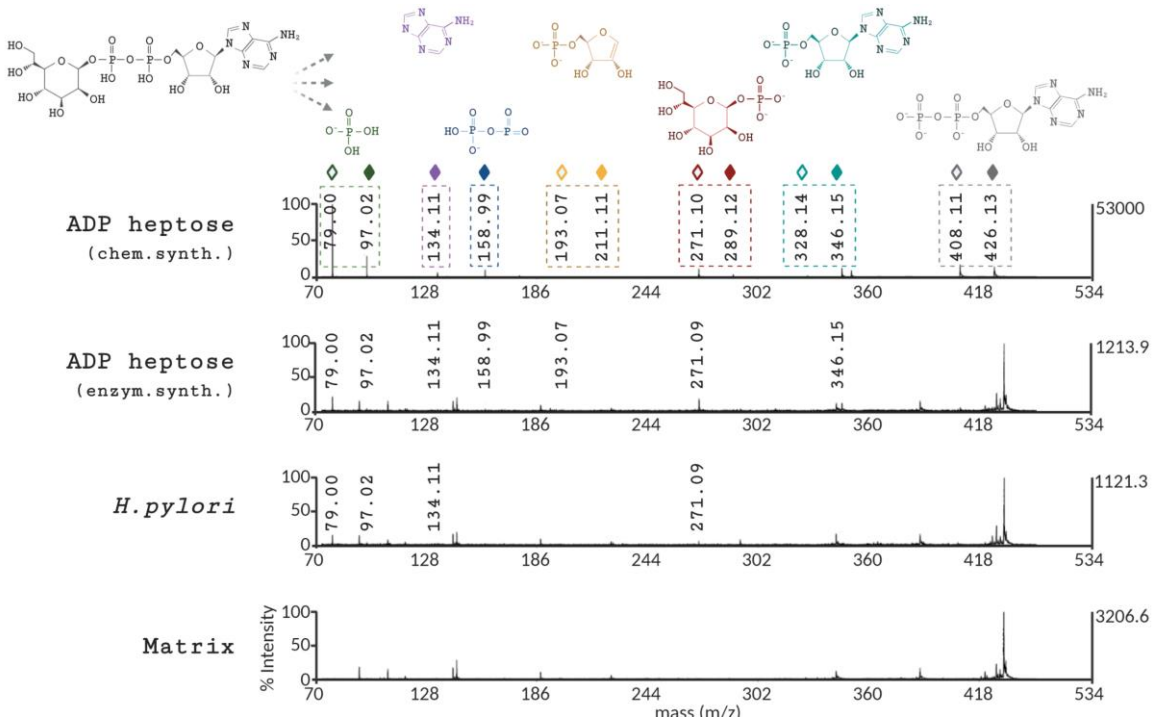


Figure 5.7 | ADP heptose is compound with mass m/z 618 [M-H]- in *H. pylori*
MALDI TOF MSMS spectra, *m/z* 618 $[M-H]^-$ of ADP heptose from chemical and enzymatical synthesis and NF-κB activating RP-UPLC fraction from *H. pylori* SPE. Matrix spectrum as control. Molecules for precursor and fragment ions are illustrated above the chromatograms: ADP heptose: black, phosphate: green, adenine: purple, pyrophosphate: dark blue, ribose-phosphate: yellow, heptose-phosphate: red, AMP: turquois, ADP: grey. For the respectively colored molecule, filled diamond shows *m/z* of ion and empty diamond corresponds to water-eliminated form. Experiment performed by Dr. Robert Hurwitz.

Taken together, in UPLC-separated fraction from *H. pylori* SPEs which were identified to activate NF-κB, it was not possible to detect HBP. However, the final product of the synthesis pathway, ADP heptose was found. To test the stimulating ability of the ADP heptose isomers, chemical and enzymatical synthesized compounds were transfected into reporter cells and relative NF-κB activation was assessed (Figure 5.8 [A]). For both isomers and synthesis methods, induction was observed for 10 µM. The final form, ADP-L-glycero-D-manno heptose showed a higher induction and was used for subsequent experiments. To test, if the concentration of up to 1 µM in *H. pylori* lysates are capable to stimulate activation, a titration was performed, revealing for 0.1 µM a 5 % and for 0.5 µM a 40 % NF-κB

activation compared to not treated cells. For HBP, the ten-times higher concentration of 5 µM induced a 5 % activation (Figure 5.8 [B]). This confirms that ADP heptose is a much more potent inducer for NF-κB than HBP. To conclude, concentration of up to 1 µM ADP heptose which were found in *H. pylori* lysates are sufficient to mediate NF-κB signaling, whereas the detected HBP concentration of up to 0.1 µM are not enough to trigger host cell response.

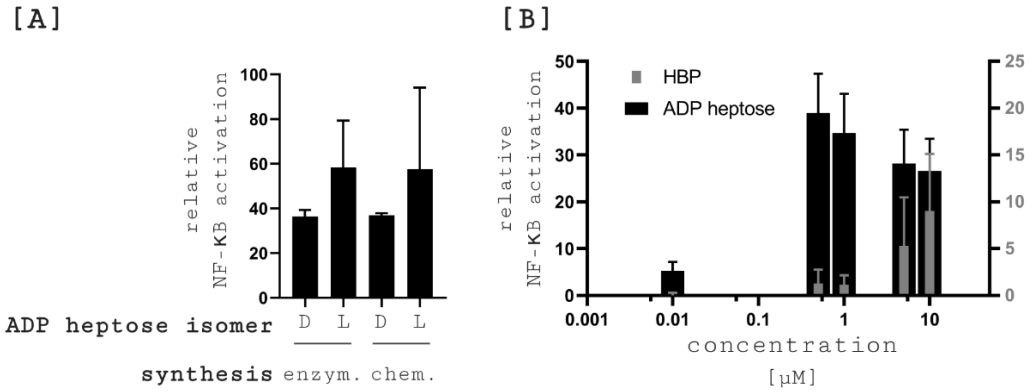


Figure 5.8 | NF-κB is activated by ADP heptose
Sugar samples were introduced into AGS NF-κB luciferase reporter cells by lipofection. Activation was measured after 3 h of treatment by luminescence of a given sample, relative to the signal of untreated cells. [A] 10 µM of ADP heptose isomers from enzymatical and chemical synthesis were used. [B] Titration of ADP-L-glycero-D-manno heptose and HBP resulting in the following concentration in µM: 0.01, 0.5, 1, 10. Y-axis labeling for ADP heptose on left side in black, for HBP on right side in grey. Experiments performed by Jan Traulsen.

5.1.4. ADP heptose/ALPK1 axis leads to PD-L1 upregulating

To summarize, the LPS synthesis metabolite ADP heptose was identified in *H. pylori* to be causative for early NF- κB response and bacteria, deficient for the sugar synthesis enzyme RfaE displayed significantly reduced capacity for PD-L1 induction compared with wt bacteria. For the immune checkpoint regulator PD-L1, a binding site (nt -610 to -601) was found to be LPS-inducible in monocytes (Huang, Wen et al. 2013). Similarly, expression was observed for the gastric cell line AGS upon LPS treatment (Li, Xia et al. 2018). Next, the signaling by LPS, ADP heptose and the precursor HBP was compared. AGS were extracellularly treated and transfected with different compound concentration and relative RNA expression was analyzed (Figure 5.9). ADP heptose was the only stimuli inducing

gene expression in both treatment procedures indicating another mode of activation for ADP heptose. Elevated levels of IL-8 and PD-L1 were observed. Transfected HBP led to chemokine but not immune ligand upregulation. Irrespective of concentration and treatment procedure, in AGS, LPS did neither induce IL-8, nor PD-L1 gene expression 3h post treatment. Taken together, this clearly identified ADP heptose as the component of the ADP heptose synthesis pathway, responsible for rapid NF-κB and PD-L1 induction in *H. pylori* lysates.

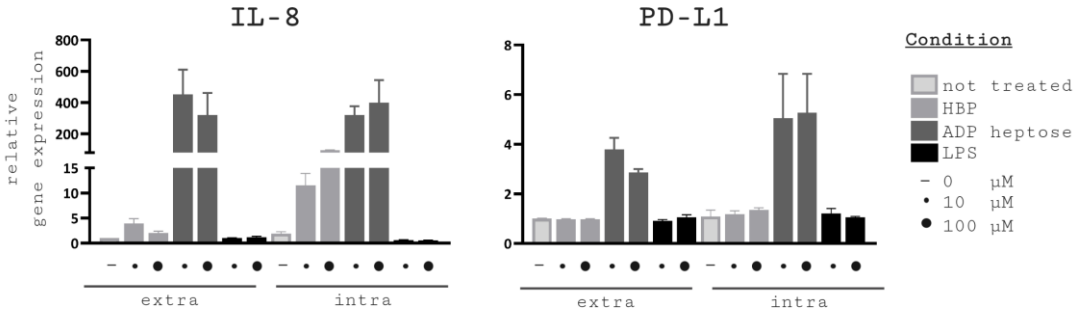


Figure 5.9 | PD-L1 is induced by ADP heptose but neither HBP, nor LPS
AGS were extracellularly treated and transfected with 10 and 100 µM of ADP heptose, HBP or LPS. Treatment was stopped after 3h. Relative mRNA expression was assessed via qRT-PCR and normalized to untreated sample. [A.] Relative IL-8 gene induction. [B.] Relative PD-L1 gene induction.

For *H. pylori* infection, the kinase ALPK1 was demonstrated to activate RfaE-specific NF-κB (Zimmermann, Pfannkuch et al. 2017). To test, if ALPK1-driven response is similarly causative for PD-L1 induction in acute infection, AGS wildtype cells and AGS cells, harboring a knockout/KO for ALPK1 were exposed to *H. pylori* for 3 h and 24 h, covering both induction peaks (Figure 5.10 [A]). In cells, deficient for the kinase, 3 h post infection, upregulation of the immune regulator was reduced compared to infected AGS wt, pointing towards an ALPK1 dependent PD-L1 upregulation. This effect is diminished in prolonged infection with similar regulation for both, wt and KO cells 24 h post infection. As inflammation regulator, PD-L1 expression can be stimulated by various cytokines. To test the ALPK1-dependency, KO cells were treated with NF-κB inducer TNFα, IRF1/STAT1 signaling molecule IFNβ and the STAT3 inducer IL-11 [Figure 5.10 [B]). No change in PD-L1 inducing capacity is observed between KO AGS and wt AGS, showing that cytokine-mediated activation is ALPK1 independent.

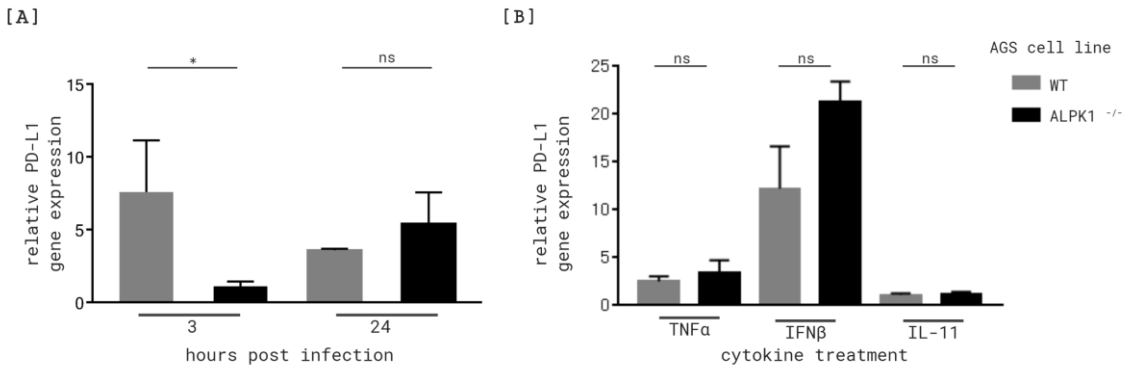


Figure 5.10 | ALPK1 is necessary for acute PD-L1 response
qRT PCR results for PD-L1 mRNA expression in infected or cytokine treated AGS WT (grey) and ALPK1 KO (black). Experiment were performed in at least three independent replicates with the exception of IL-11 (2 replicates). Statistical analysis was performed using Student´s *t*-test (*, $p \leq 0.05$). [A] Cells were infected with *H. pylori* MOI50 for 3 h and 24 h. Relative expression was assessed in respect to gene expression at timepoint 0. [B] AGS WT and AGS, deficient for ALPK1 (ALPK1$^{-/-}$) were treated with 10 µM of the following cytokines for three hours: TNFα, IFNβ, IL-11. Relative expression was assessed in respect to gene expression of untreated sample.

To summarize, as fast induction of PD-L1 in response to *H. pylori* depends on activity of ALPK1 and the bacterial compound ADP heptose.

5.1.5. ADP heptose / ALPK1 is a major regulator for early response to infection

To characterize the newly discovered ADP heptose / ALPK1 signaling axis, a global experimental approach was used, where wildtype AGS and AGS bearing an ALPK1 KO were treated with the sugar ADP heptose or infected with the Gram-negative bacterium *H. pylori.* Gene expression was assessed using two-color microarrays. In this technique, two different samples are hybridized on a single microarray and fluorescently labeled with the cyanine-based dyes Cy3 or Cy5. To increase statistical significance of the outcome, two biological replicates were tested and a dye swap was performed to eliminate technical errors like differences in dye incorporation efficiency. For hybridization design, six different sample comparisons were used (Figure 5.11 [A]), resulting in expression fold change (FC) and probability (p-value) for the tested probes on the microarray. To interpret the results, expression of predefined genes of interest were assessed and as unbiased analysis approaches, Venn diagrams and gene set enrichment analysis (GSEA)

were applied. Venn diagrams compare a certain set of microarray results with each other in regard to gene regulation pattern, sorted for genes with p-value ≤ 0.05 and log(FC) ≤ 0.6. GSEAs are used to categorize deregulated genes within well-defined gene sets. To increase the significance of the used genes, the false-discovery rate (FDR) Benjamini-Hochberg correction was used prior to GSEA.

1.) ADP heptose signaling depends on ALPK1

To study the effect of ADP heptose treatment on ALPK1 a Venn diagram was used, comparing treatment in wt cells and KO cells (Figure 5.11 [B]). The diagram revealed a total number of 1,673 deregulated genes in ADP heptose treated wt cells. Sugar-treated ALPK1 KO cells on the other hand showed (without FDR) only 244 deregulated cells. Based on this, it can be concluded, that ADP heptose exerts its function mainly, if not exclusively, by ALPK1. This is supported by the fact, that only two genes are commonly regulated between sugar-treated wildtype and KO cells. Commonly deregulated pathways would include a large number of genes, hinting towards a false-positive regulation of the found genes. When the false-discovery rate adjustment using Benjamini-Hochberg procedure was applied, no genes were found in ADP heptose treated KO cells.

2.) ALPK1 KO leads to impaired immunity

To check for implication in the PD-L1:PD-1 regulation, expression for both genes was evaluated (Figure 5.11 [C]). Supporting previous results, for wt AGS sugar treatment (I) and infection (IV) led to increase of the ligand PD-L1, whereas the KO for ALPK1 diminished the effect (II and V). Unexpectedly, gene regulation for the receptor PD-1 was influenced by the KO (III), leading to a downregulation. The PD-L1:PD-1 axis is necessary for a functioning immunity. To estimate the global consequences of sugar treatment or kinase KO on the immunological level, GSEA was performed and analyzed in this regard. The immunologic signature collection, ImmuneSigDB, is a collection of around 5,000 gene sets derived from approximately 400 immunological studies, including cell types, states, and perturbations within the immune system (Godec, Tan et al. 2016). For each microarray result, the number of regulated gene sets belonging to ImmuneSigDB was blotted, separately for downregulated and upregulated gene sets (Figure

5.11 [D]). Compared to wt condition, ALPK1 KO results showed for all settings (II to I, V to IV) a lower number for regulated gene sets, highlighting ALPK1s role in immunity. This is supported by the large number of downregulated gene sets found in infected KO cells compared with infected wt cells (VI).

3.) ADP heptose/ALPK1 axis is involved in a variety of processes

To summarize, ALPK1 deficiency is linked with a disturbed and predominantly reduced immunological phenotype in homeostasis, ADP heptose treatment and *H. pylori* infection. To set this in a wider range of biological processes, the gene set collection "hallmark" of the Molecular Signatures Database (MSigDB) was used (Figure 5.11 [E]). The collection of 50 hallmarks condenses information from original over 8,000 gene sets to around 4,000 gene sets by computational procedure with manual expert curation, thereby reducing variation and redundancy (Liberzon, Birger et al. 2015). From the compendium of 500 hallmark sets, 35 were found to be deregulated. For ADP heptose treated wildtype cells (I), a predominantly activating phenotype is visible with highest magnitude for "TNFα signaling via NF-κB", highlighting the importance of the NF-κB pathway. No change was observed for ADP heptose treated KO cells (II), as no deregulated genes were there after FDR-correction. Hallmark gene sets were only marginally affected by the introduction of the ALPK1 KO compared to wt cells (III). Three hours of infection with *H. pylori* (IV) showed, similar to the sugar-treatment, a predominantly upregulating phenotype, with the highest effect size and p-value for "TNFα signaling via NF-κB". From the 28 hallmarks, which are mainly upregulated, 18 were also found to be upregulated in (I), indicating that the phenotype after 3 h of infection is mainly ADP heptose driven. Infected KO cells (V) show upregulation of 8 hallmarks with low effect size and p value, highlighting the importance of ALPK1. This is supported by the mainly downregulating phenotype, found for infected KOs compared to infected wt cells (VI).

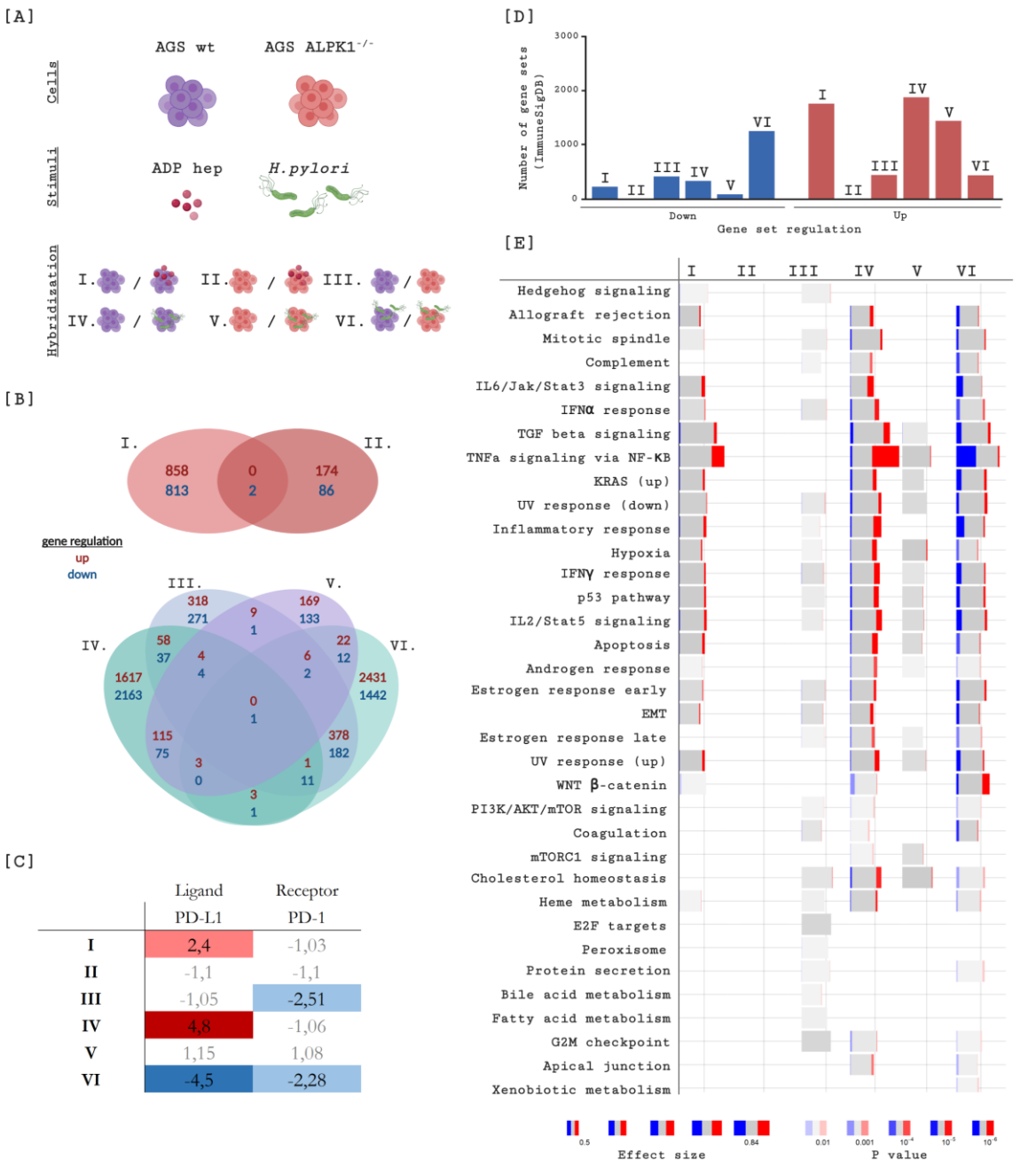


Figure 5.11 | ADP heptose / ALPK1 is major signaling axis in early infection

[A] Cells and stimuli used in the microarray and hybridization design with six different comparisons. Roman numeral labeling is used for following figures. [B] Venn diagrams summarizing microarray results. For the following figures, the color red indicates upregulation and blue downregulation. [C] Fold change for PD-L1 and PD-1 in all comparisons. [D] Number of deregulated gene sets belonging to the ImmuneSigDB. [E] Deregulated gene sets of MSigDB "hallmark". Sugar treatment and infection was performed by Felix Müller. Microarray data analysis was performed by Hans Joachim Mollenkopf.

5.2. Late *H. pylori* infection response

Regulation of the immune checkpoint protein PD-L1 is a multifactorial process. Even in an basic infection model of such as an *in vitro* infection assay where immune cells and connection to other tissue are absent, two distinct regulating mechanism could be observed (Figure 5.1). The first peak, 3 h post infection could be delineated to the ADP heptose / ALPK1 signaling axis, whereas the second peak was shown to be ALPK1- independent as similar induction levels were observed for 24 h-infected KO and wt cells (Figure 5.10).

5.2.1. Host directed metabolic activation is crucial for PD-L1 induction

To elucidate the factors causative for late PD-L1 induction, AGS were infected with *H. pylori* wt and bacterial mutants, lacking important bacterial virulence factors VacA, CagA, cagPAI or CGT (Figure 5.12 [A]). After 24 h, no significant change in relative gene expression compared to wt infection was observed for the mutants Δ*vacA* and Δ*cagA*. Bacteria, deficient for cagPAI showed similarly no hampered PD-L1 induction, providing further proof, that the ADP heptose / ALPK1 axis is not regulatory involved in lade regulation of the ligand. Infection with Δ*cgt* mutant bacteria led to a significant reduced induction of PD-L1 compared to wt infection. These bacteria lacking the cholesterol-alpha-glycosyltransferase CGT which catalyzes the first reaction in a process of *H. pylori* to extract cholesterol from host membranes in order to incorporate it into its membrane (Figure 5.12 [B]) (Lebrun, Wunder et al. 2006). Several studies provided evidence that the extracting mechanism is linked with host immune evasion (Wunder, Churin et al. 2006, Morey, Pfannkuch et al. 2018). To compensate for the loss of membranous sterol, new synthesis of cholesterol takes place within infected cells (*unpublished data*) and a deregulation of metabolic processes was suspected.

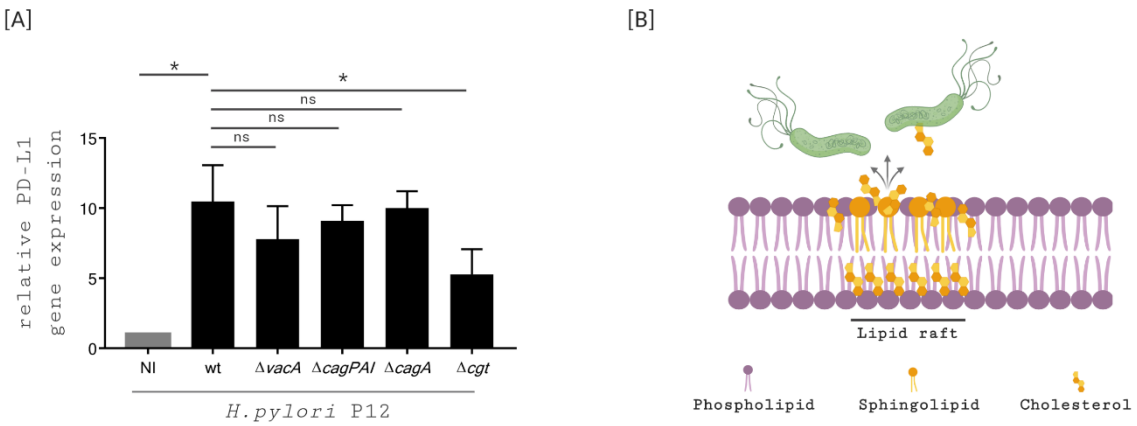


Figure 5.12 | Cholesterol extracting activity is linked with PD-L1 induction
[A] AGS infected with H. pylori, MOI 50 wildtype (wt), ΔvacA, ΔcagPAI, ΔcagA, Δcgt or left uninfected for 24h. Relative PD-L1 mRNA expression was assessed via qRT-PCR in respect to gene expression of uninfected sample. Statistical analysis was performed using Student´s t-test (*, $p \leq 0.05$). [B] Illustration of cholesterol depletion by *H. pylori*. In lipid rafts, high contents of cholesterol and sphingolipids are found.

Microarray data of human gastric primary cells, infected with *H. pylori* wt and *H. pylori* Δ*cgt* was analyzed accordingly, with help of the Kyoto Encyclopedia of Genes and Genomes / KEGG database to identify genes, encoding for proteins involved in metabolic processes. A global map of metabolic pathways, compromising about 120 metabolic pathways from large-scale molecular data sets (Kanehisa, Araki et al. 2008), was used. Comparison with the list of deregulated genes from wt infected primary cells from the microarray showed that a large set of genes involved in metabolic pathways is significantly deregulated in the course of long infection (Figure 5.13 [A]). To elucidate the effect caused by the presence of CGT, a volcano blot was generated including both, genes which are deregulated due to wt infection and due to Δ*cgt* mutant infection (figure 13 [B]). A remarkable reduction in gene number can be noticed for Δ*cgt* infection, highlighting the impact of cholesterol extraction on host metabolic shift.

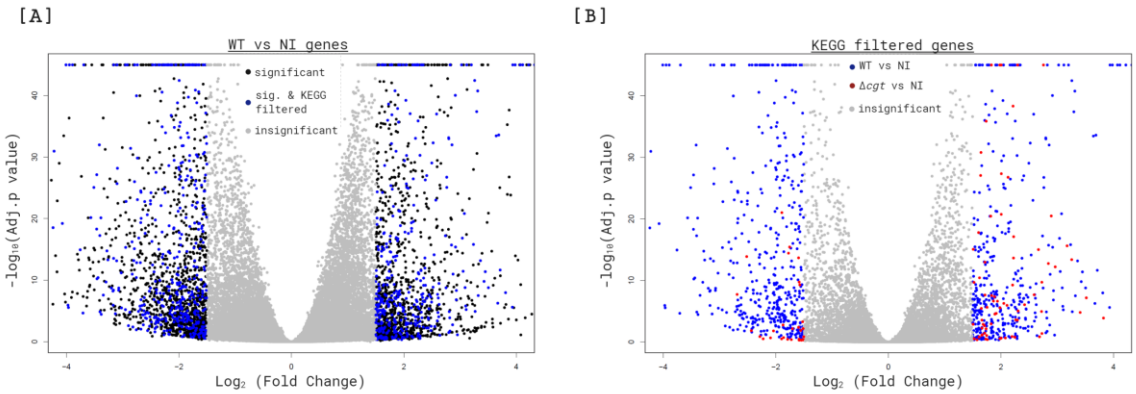


Figure 5.13 | Bacteria induced cholesterol extraction in host cells leads to metabolic deregulation

Microarray data of primary human gastric cells, uninfected or infected with *H pylori* wt or Δ*cgt* for 17 h (Morey, Pfannkuch et al. 2018). [A] Volcano plot of deregulated genes in *H pylori* wt infected cells compared to uninfected condition. Insignificant genes (fold change ≤1.5) in grey, genes contained in KEGG Pathway Metabolism in blue, rest black. [B] Volcano plot of deregulated genes contained in KEGG Pathway Metabolism. Insignificant genes (fold change ≤1.5) in grey, genes deregulated by *H pylori* wt infection blue, genes deregulated by *H. pylori* Δ*cgt* infection red.

To decipher the deregulated pathways, the top gene sets of the KEGG database, based on the GSEA score were blotted, revealing “Glycolysis / Gluconeogenesis” as the top hit. Glycolysis is the process in which glucose is metabolized to pyruvate. Subsequently, under aerobic conditions, in aerobic organism and tissues, pyruvate is usually oxidatively metabolized to the acetyl group of acetyl-coenzyme A. In the tricarboxylic acid (TCA) cycle it is completely oxidized to CO_2, releasing electrons which are passed to O_2 through a specific chain of carriers in the mitochondria, forming H_2O and the synthesis of ATP in a process known as oxidative phosphorylation. Under low oxygen conditions/hypoxia or anaerobic conditions, pyruvate is reduced to organic acids or alcohol, such as lactate or acetate in a process known as fermentation (Nelson 2005). Compared to glycolysis, followed by TCA cycle and respiration, fermentation is less efficient in terms of ATP production. Many unicellular organisms, such as *Saccharomyces cerevisiae* rely on fermentation during proliferation, even in the presence of oxygen (Lunt and Vander Heiden 2011). Most cells of multicellular organism have a quiescent metabolic state in nonproliferating and differentiated condition. Proliferating cells on the other hand, like activated lymphocytes or embryonic stem cells perform

fermentation to generate ATP, regardless of oxygen availability (Escoll and Buchrieser 2018). This process, termed aerobic glycolysis, was first described by Otto Warburg in cancer cells which have a high glucose demand a lactate production (Warburg 1924, Warburg 1956). Besides the described catabolic fate, pyruvate represents a precursor for many anabolic processes, such as the synthesis of amino acid or fatty acid. Like glycolysis, gluconeogenesis is a catabolic process by which glucose is however produced from non-carbohydrate precursors. It permits the maintenance of blood glucose levels under energy-demanding conditions such as low-glucose diets, fasting and intensive exercise and is restricted to the liver, kidney, muscle and intestine (reviewed in (Wang and Dong 2019)).

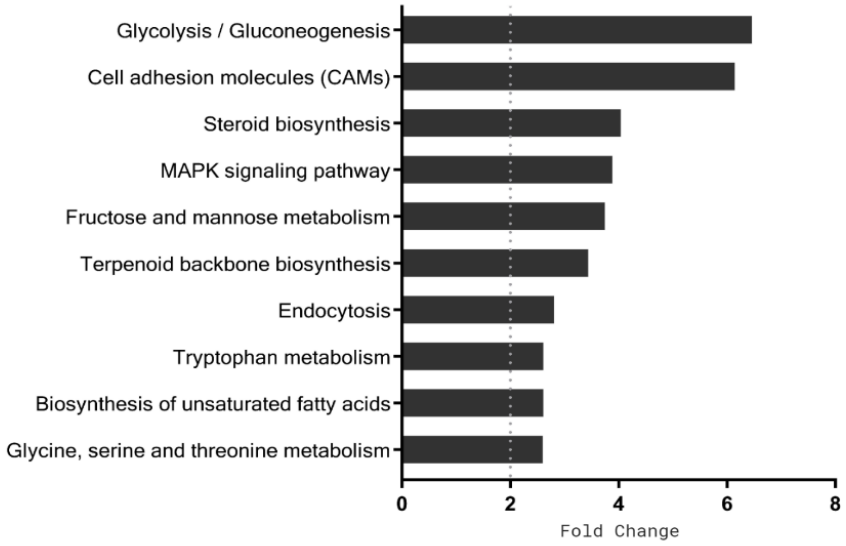


Figure 5.14 | Deregulated metabolic pathways upon *H.pylori* infection
Based on microarray data of HGAT, infected with *H pylori* wt for 17 h (Morey, Pfannkuch et al. 2018). List of Top10 deregulated pathways according to GSEA using the KEGG database.

To test, if the changed metabolism has an influence on PD-L1 induction, infected cells were treated with clotrimazole (CLT). The imidazole is best known for its antimycotic potency against fungal infections such as caused by *Candida albicans*. Its action inhibits biosynthesis of ergostol, the main sterol in fungal cell membranes. It interferes with the calcium and kalium movement across plasma membranes and

treatment potency was shown for several cancers, such as melanoma or breast cancer (Benzaquen, Brugnara et al. 1995, Coelho, Calaça Ide et al. 2011, Crowley and Gallagher 2014). The anticancer effects are linked with a decrease in glycolytic flux by inhibition of key enzymes (Meira, Marinho-Carvalho et al. 2005, Zancan, Rosas et al. 2007).

AGS infected with *H. pylori* and simultaneously treated with clotrimazole showed a significant decrease in PD-L1 induction compared to untreated, infected cells (Figure 5.15 [A]). To verify that the treatment has no influence of *H. pylori*, bacterial fitness was assessed by plating of bacteria which were subjected to CTL treatment (Figure 5.15 [B]). No significant difference between untreated and treated bacteria was observed. This shows that the decline in PD-L1 induction is not caused by decreased bacterial infectivity but rather CTLs action on the host cell.

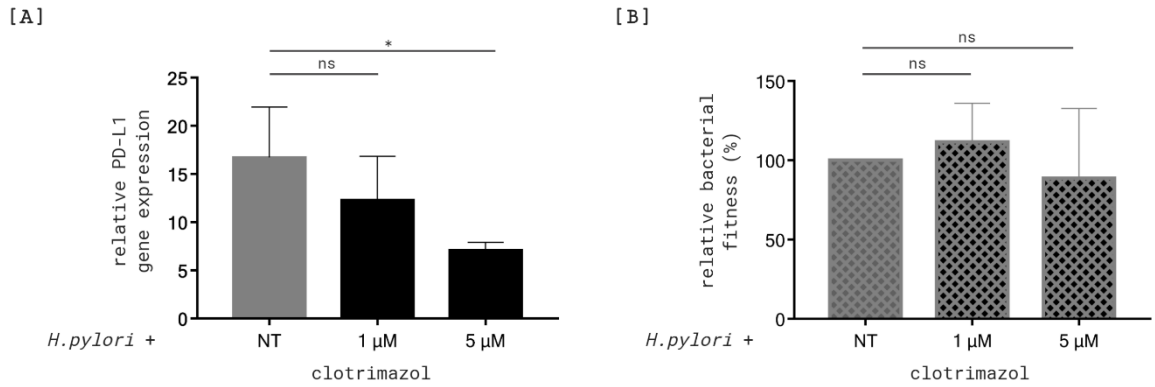


Figure 5.15 | Downregulation of glycolysis interferes with PD-L1 upregulation
AGS were simultaneously infected with *H. pylori*, treated with clotrimazole (1 µM, 5 µM) or left untreated for 24 h. Statistical analysis was performed using Student´s *t*-test (*, $p \leq 0.05$) with three independent replicates. [A] qRT PCR results for PD-L1 mRNA expression. Relative expression was assessed in respect to gene expression in untreated/uninfected condition. [B] Bacterial supernatant was collected and plated. Colony number was assessed. Clotrimazole treated bacteria were compared to untreated sample.

5.2.2. Activity of hexokinase enzymes regulate PD-L1

KEGG analysis revealed glycolysis and gluconeogenesis as the most prominent deregulated pathways. Next aim was to determine, which of them is upregulated. While glycolysis is the process, converting glucose to pyruvate, gluconeogenesis

describes the generation of glucose from non-carbohydrate carbon substrates such as pyruvate, lactate, glycerol, and glucogenic amino acids. Although both pathways share similarities, one is not simply the reverse of the other and divergences are found in the initial and final steps. The pathways share seven of their enzyme-catalyzed reactions and are coordinated that within a cell one is rather inactive with the other being highly active. To decipher which is active due to *H. pylori* infection, the deregulated genes were compared (Figure 5.16 [A] and [B]). Upregulated genes are hexokinase (HK), glucose-6-phosphate isomerase (GPI), aldolase (ALDO), triose-phosphate isomerase (TPI), phosphoglycerate kinase (PGK) and enolase (ENO). Out of these, HK represents a protein, designated exclusively for the glycolysis pathway by catalyzing phosphorylation of glycose and thereby trapping it within the cell. The produced glucose-6-phosphate is used for energy production via glycolysis and a precursor, needed for glycogenesis, pentose phosphate pathway and hexosamine biosynthetic pathway. Four different isozymes of HK could be separated by ion exchange chromatography, referred to type I – IV / HK1 – HK4 (Figure 5.16 [C]) (González, Ureta et al. 1964, Katzen and Schimke 1965). In contrast to the 50 kDa HK3, the 100 kDa type I – III isozymes display an internal sequence repetition which is thought to originate from fusion or duplication of an ancestral 50kDa HK (Bork, Sander et al. 1993, Cárdenas, Cornish-Bowden et al. 1998).

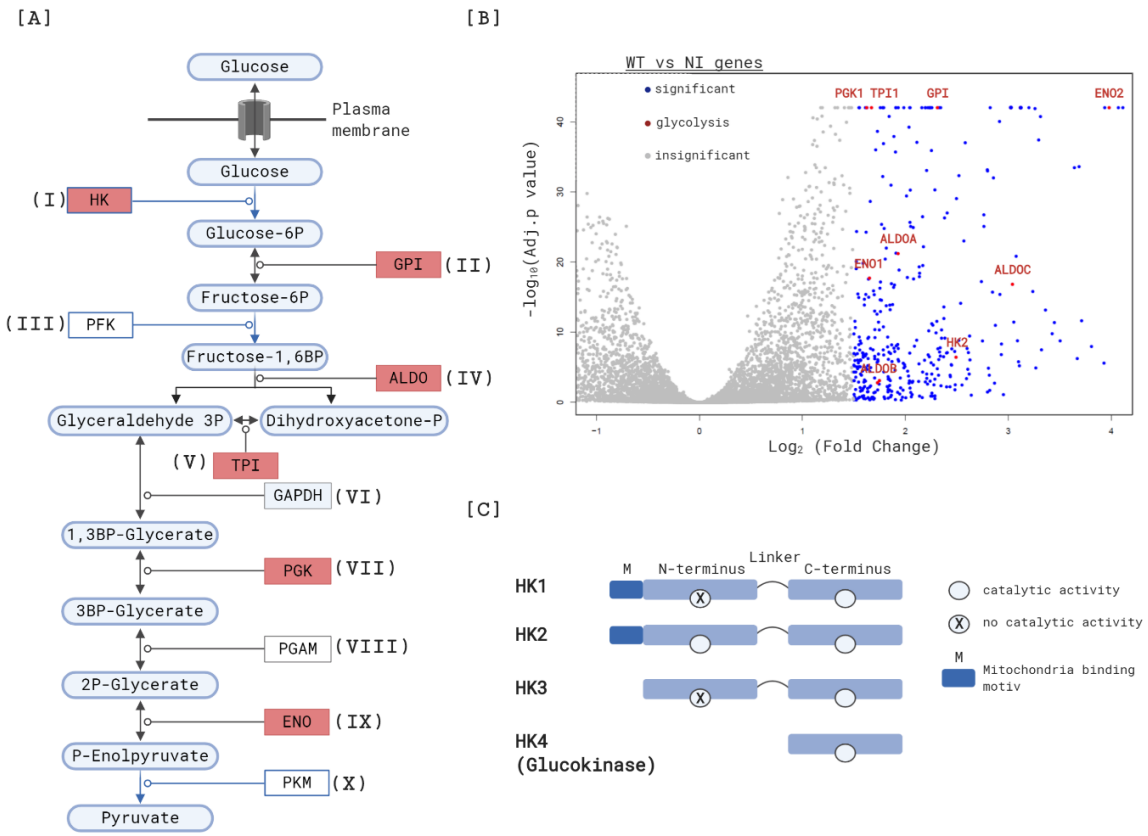


Figure 5.16 | Glycolysis, not Gluconeogenesis is upregulated
[A] Schematic representation of glycolysis pathway with genes (red), deregulated in primary gastric epithelial cells, infected with H. pylori for 17 hours. Steps: (I) Glucose is phosphorylated by hexokinase to glucose-6 phosphate. (II) Through isomerization via a phospho-glucose isomerase, a ketose is formed a carbonyl oxygen rearrangement. (III) Phosphorylation by phosphofructokinase to fructose 1,6 bisphosphate. (IV) Out of the six-carbon sugar, two three-carbon molecules are cleaved by aldolases into dihydroxyacetone phosphate and glyceraldehyde 3-phosphate. (V) The latter can proceed, while the other sugar gets isomerized by a triose phosphate isomerase. (VI) An anhydride linkage to phosphate is formed by glyceraldehyde 3-phosphate dehydrogenase, resulting in two molecules of 1,3-bisphosphoglycerate. (VII) Phosphoglycerate kinase activity leads to phosphate transfer, forming 3-phosphoglycerate. (VIII) The phosphate ester linkage is moved from carbon 3 to 2 by phosphoglycerate mutase, resulting in 2-phosphoglycerate. (IX) An enolase removes water, creating phosphoenolpyruvate. (X) The phosphate group is transferred by a pyruvate kinase, creating the end product pyruvate. Adapted from (Alberts 2014) Blue arrows indicate glycolysis specificity. [B] Volcano Plot, showing genes, involved in metabolism, that are deregulated in primary gastric epithelial cells, infected with H. pylori for 17 hours. Gene names correspond to nearest red dot and belong to glycolysis pathway. [C] Adapted from (Roberts and Miyamoto 2015)

Since HK, which catalyzes the first step of glycolysis, was found to be upregulated, it can be assumed that not gluconeogenesis but glycolysis is upregulated due to *H. pylori* infection. In the microarray, isoform II was found to be upregulated. HK2 is the only form, in which the catalytic activity is preserved in both domains while otherwise being restricted to the carboxyl terminal domain. Throughout the body,

the isoforms are selectively and differentially expressed: HK1 is ubiquitously expressed and the main form of brain tissue. HK2 is much more limited with expression restricted to tissues such as cardiac muscle, skeletal or adipose tissue. HK3 is ubiquitously expressed at low levels and HK4 is primarily restricted to the pancreas and liver (Roberts and Miyamoto 2015).

To validate the observation of HK2 expression and since HK1 should be the predominant isoform in these non-insulin-sensitive cells, gene expression for both enzymes was followed over a time of 48 h of *H. pylori* infection in AGS (Figure 5.17 [A]). For both a time-dependent increase can be noted with peak levels 12 h post infection for HK1 and 24 h post infection for HK2. Due to its high catalytic ability among the isoform, expression is found in various cancer tissues (Patra, Wang et al. 2013, Liu, Li et al. 2019). AGS are a cancer cell line and to eliminate that this is causative for HK2 upregulation, expression was checked in infected human gastric primary cells, originating from three independent donors (Figure 5.17 [B]). It was found to be significantly increased due to wildtype infection, while bacteria deficient for *CGT* displayed a significant reduced ability to upregulate HK2 compared to *H. pylori* wt.

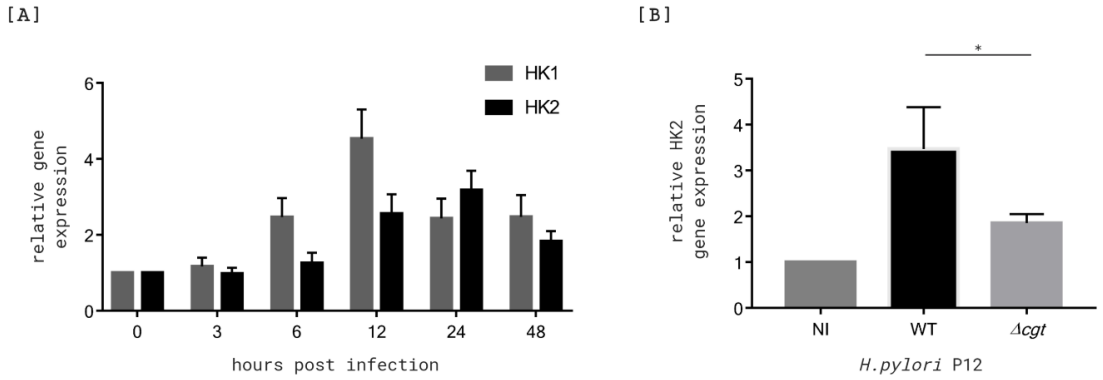


Figure 5.17 | In AGS and primary cells, HK2 is upregulated in response to infection
qRT PCR results for cells infected with *H. pylori*. Statistical analysis was performed using Student´s *t*-test (*, $p \leq 0.05$) with three independent replicates. [A] Infection was performed with wt bacteria and stopped after 3 h, 6 h, 12 h, 24 h, 48 h. Relative HK1 and HK2 mRNA expression was assessed in respect to gene expression at timepoint 0. [B] Infection was performed with wt or Δ*cgt* bacteria and stopped after 24 h. Relative HK2 mRNA expression was assessed in respect to noninfected sample.

To evaluate the role of specific enzymes of the glycolysis pathway in the upregulation mechanism of PD-L1, a knockdown was performed, targeting

proteins, catalyzing the first as well as the last step of glycolysis (Figure 5.18**)**. The initial step is triggered by hexokinase. Isoform HK1 and HK2 were tested, as both proteins were found to be influenced in the infection process (Figure 5.17 [A]). The last step of glycolysis is driven by the enzyme pyruvate kinase M (PKM) of which the two splice variants PKM1 and PKM2 exist. Both kinases convert phosphoenolpyruvate to pyruvate. PKM2 was tested as publication provided evidence for the involvement in PD-L1 regulation whereas PKM1 was published to be linked with immune cell activation (Palsson-McDermott, Dyck et al. 2017, Watanabe, Shirai et al. 2017), an event speaking against the involvement of the immune cell inhibitor PD-L1.

To ensure equal loading per well, actin was used as loading control and showed comparable levels among all samples. As loading control for bacteria, the amount of the effector protein CagA was used, which shows similar levels post infection. Protein levels for tyrosine-phosphorylation (pY99) of CagA (pCagA) were used to evaluate bacterial fitness, as only living bacteria are capable to translocate the effector protein CagA into the cell, where it get phosphorylated. Comparable levels were observed for all infected specimen. In control samples, an increase in PD-L1 protein expression can clearly be observed after 24 h of *H. pylori* infection. The HK isoforms show a distinct protein pattern upon infection. In the control samples, HK1 decreases whereas HK2 increases 24 h post infection, strengthen the hypothesis of a HK switch in the infection course. HK1 KD led to absence of the HK1-specific but also HK2- and PKM2-specific band. The induced KD led to a significant PD-L1 protein reduction post infection. Cells, harboring a PKM2 KD showed a similar PD-L1 expression level as control cells, providing no evidence for a PKM2-driven regulation. The KD efficiency was validated by the absence of the PKM2-specific protein band. For the HK2 KD, a panel of different siRNAs was tested (a-d) for validation:

siRNA (a) worked as protein levels for the target were downregulated prior and post infection. The KD did not influence HK1 expression and showed a downregulation of PD-L1 upon infection, pointing towards a HK2-driven regulatory mechanism. siRNA (b) increased target protein post infection and HK1 was likewise

effected. Usage of this siRNA promoted expression of PD-L1. siRNA (c) had no effect on the target as well as the isoform HK1. PD-L1 protein level were not different from control samples.siRNA (d) showed not an optimal KD efficiency, as a light HK2 protein band still appears post infection. HK1 protein level is not effected. PD-L1 protein level is reduced compared to control sample.

To sum up, siRNA-mediated HK1 KD lead to decrease in PD-L1 expression but was not specific as expression of the glycolytic enzymes HK2 and PKM2 were also influenced. A subsequent KD of PKM2 did not alter PD-L1 expression, showing that this enzyme is not regulatory involved. siRNA successfully targeting HK2 (a and d) led to reduced PD-L1 protein expression post infection but not prior to infection, pointing towards a HK2-specific role in PD-L1 upregulation. siRNA mediated aberrant HK2 expression potentiated PD-L1 expression post infection, strengthening the of HK2. To finally validate the involvement, next aim was to generate a stable HK2 KO cell line. While a RNAi-mediated knockdown acts on mRNA level, a knockout silences the protein on DNA level, ensuring permanent and not just transient loss-of function for further tests.

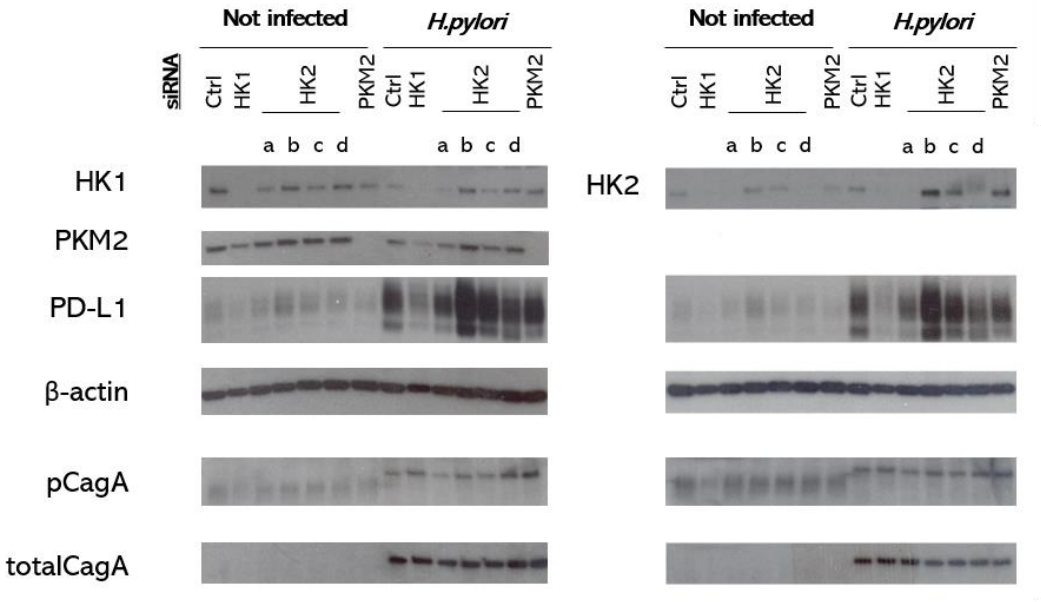


Figure 5.18 | Hexokinases are implied in infection-induced PD-L1 upregulation
Western blot analysis of siRNA-mediated knockdowns. Cells were transfected while seeding and two days later infected with *H. pylori* MOI 50 for 24 h or left uninfected. Samples were analyzed by using antibodies against HK2, PD-L1, actin, PKM2, phospho-tyrosine 99 (pCagA) and total CagA. Representative of at least two replicates.

5.2.3. Stable HK2 knockout cells display diminished capacity to induce PD-L1

In order to generate a knockout cell line, the ancient defense system CRISPR/Cas9 was introduced into the AGS cell system, using a one-vector lentiviral plasmid system (lentiCRISPRv2), containing an expression cassette for a chimeric sgRNA and one for *Streptococcus pyogenes* Cas9 nuclease.

The generation of KOs, derived from a single clone, requires a five step procedure (Figure 5.19 [A]). Of substantial importance is the first step, the selection of the target site within the gene of interest. A promiscuous binding within the genome will result in off-target mutagenesis and many sgRNAs were shown to lead to a low cutting rate. To address this challenge, the online tool CHOPCHOP was used to design HK2-specific target sequences (Figure 5.19 [B]). The web-based tool allows to design CRISPR/Cas9 target sites based on large scale studies and calculates off-target potential. (Labun, Montague et al. 2016) Three different target sites were selected, located on exon 9, 7 and 1 with the trinucleotides 5'-CGG-3' and 5'-GGG-3' as PAMs since the used vector system contains a *Streptococcus pyogenes* Cas9 nuclease, requiring a 5'-NGG-3' sequence (Anders, Niewoehner et al. 2014). The sequence sites were choose, as they were highest in the ranking, vary in the exon and no off-targets were predicted.

After target sequences cloning, vector delivery into the cells and plasmid-specific selected with puromycin, the cells were FACS sorted to obtain single cells. The clones were expanded and characterized by western blot, after 24 h of infection for the ability to express HK2 and regulate PD-L1 (Figure 5.19 [C]). For all samples, the loading control actin and for the infected specimen, the bacterial control totalCagA showed comparable levels. Similarly, the bacterial fitness control pCagA showed equivalent levels, indicating that a KO of HK2 does neither affect the uptake, nor the phosphorylation of the *H. pylori* virulence factor. In AGS wt, increase of HK2 and PD-L1 protein levels can be observed post infection. With the exception of clone II.c, all clones displayed a loss of HK2. Clone I.a and II.b showed reduced PD-L1 levels in uninfected conditions and with the exception of II.c, reduced levels were also observed 24 h post infection compared to AGS wt, showing a functioning

KO. Clone II.c displayed not complete but decreased HK2 protein expression and an increase in PD-L1 protein expression post infection.

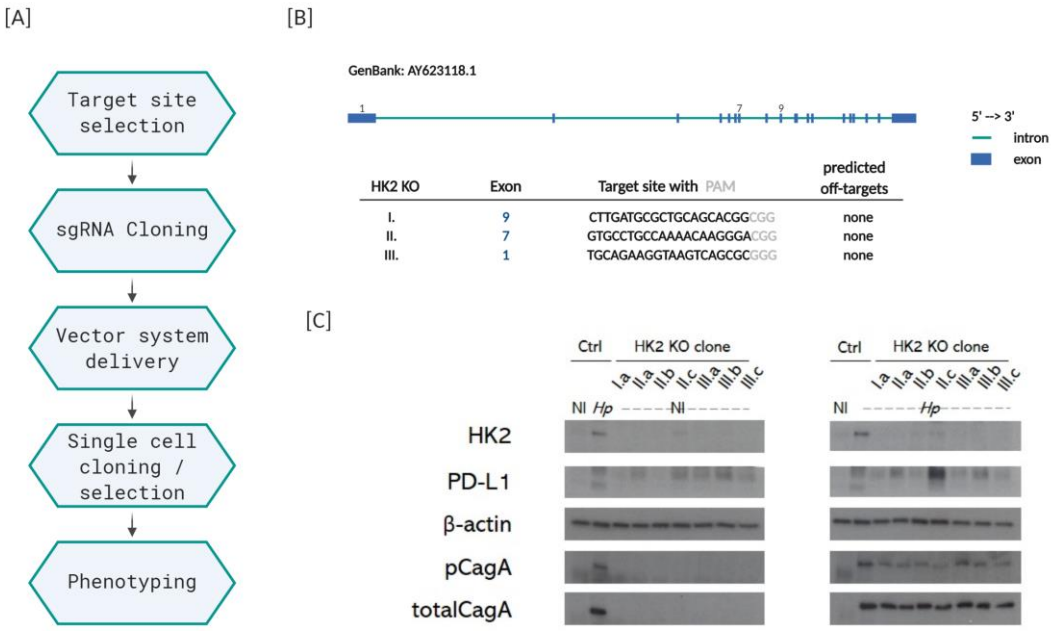


Figure 5.19 | Generation of CRISPR/Cas9 HK2 KO cell line
[A] CRISPR/Cas9 workflow. Using the ChopChop online tool, Cas9 target sites (crRNA) were selected and designed with tracr RNA (PAM) as binding scaffold for the Cas nuclease, forming the sgRNA (see [B]). sgRNAs of target sites were cloned into the guide RNA scaffold of the lentiviral plasmid lintiCRISPRv2, which contains Cas9 nuclease. [B] Selected sgRNAs, searched with ChopChop online tool (https://chopchop.cbu.uib.no/) for HK2. Sequence and location of gene specific target region, PAM sequence and predicted off-targets were calculated. [C] Western blot of HK2 KO clones. Cells were infected with *H. pylori* MOI 50 for 24 h or left uninfected. Samples were analyzed by using antibodies against HK2, PD-L1, actin, phospho-tyrosine 99 (pCagA) and total CagA.

Several HK2 KO clones were generated that display a reduced capacity to regulated PD-L1. Clone III.c, which showed no HK2 protein expression, a strong reduction in PD-L1 expression upon infection while maintaining a good growth behavior was selected for further phenotyping. The clone was generated, targeting exon 1, ensured that no short protein fragment could be generated, as it would have been possible with sgRNAs targeting later exons.

Next aim was to characterize clone III.c (following referred to as “HK2 KO”). To determine basal gene expression of the target gene, qRT-PCR was used for untreated cells and compared with AGS wt (Figure 5.20 [A]). A relative fold-change of 0.6 compared to AGS wt revealed a significant decrease but still existent mRNA

levels. A possible reason for the remaining mRNA could be found in the consequences of the DSB repair. Generation of indels can lead to in-frame mutations, leading to the generation of a transcript, detectable by qRT-PCR. The absent protein band (Figure 5.19 [C]) shows that an Indel-produced STOP-codon could have led to premature interruption of protein translation. To validate the PD-L1 inducing capacity, infected HK KO were characterized using qRT-PCR and confocal microscopy (Figure 5.20 [B] and [C]). For mRNA as well as protein expression, a reduction was noticed compared to infected AGS wt. However, PD-L1 upregulation was still observed, showing that additional or compensatory mechanism are involved in the regulation process besides HK2.

To summarize, It could be shown that a knockdown of HK2 leads to downregulation of PD-L1 gene expression and with a stable AGS knockout cell line for HK2 this observation could be verified, as a decrease in protein expression was observed. Next aim was to test the consequences of an increased glycolytic activity.

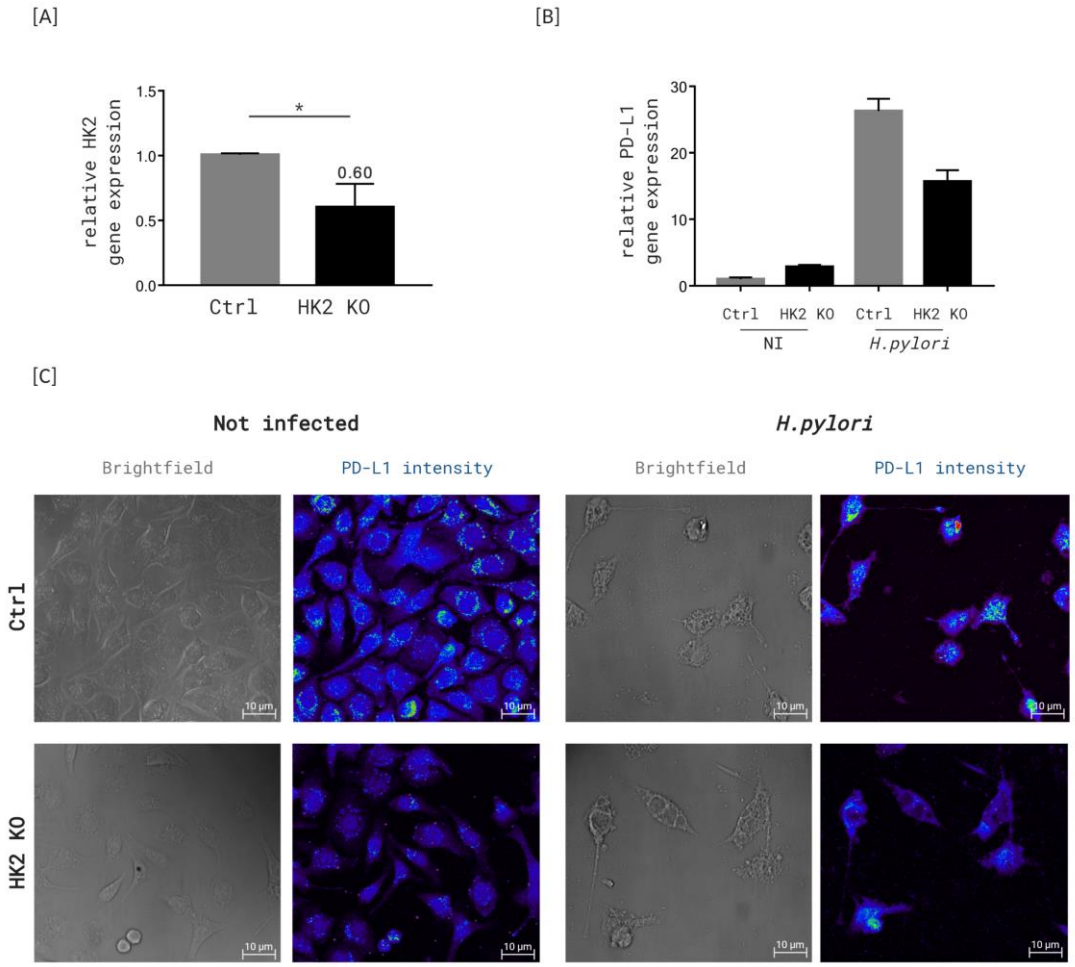


Figure 5.20 | HK2 KO impairs PD-L1 upregulation post infection
[A] HK2 mRNA expression in the monoclonal cell line III.c (AGS JAB004) relative to wildtype AGS. [B] AGS wildtype or HK2 KO were infected with *H. pylori* MOI 50 for 24PD-L1 mRNA expression was assessed relative to uninfected samples. Graph is representative of three independent experiments. [C] AGS infected with *H. pylori*, MOI 50 for 24 h. Upon infection, cells were fixed and subjected to anti-PD-L1 antibody and DAPI, and subsequently analyzed by confocal microscopy.

5.2.4. Host directed metabolic activation enhances resistance towards infection

Linked with enhanced rates of glucose uptake and glycolysis, the switch from another HK isoform to HK2 is often observed in cancer tissue. Infection with *H. pylori* leads to a similar change in expression pattern (Figure 5.17 [A] & Figure 5.18). To check if this leads to an increase of the glycolytic end product, infected cells were tested after 3 h and 24 h and pyruvate content was measured (Figure 5.21 [A]). After 3 h, no significant change was observed which is in line with the observation that upregulation of both HKs and therefore most likely metabolism in general occurs later in the course of infection. After 24 h, a significant increase in

pyruvate content was noted, showing the direct effect of the increase in glycolysis, which was observed before by gene upregulation of key enzymes (Figure 5.16 [A] and [B]). It can be concluded that infection with *H. pylori* wt leads after 24 h to a metabolic change with upregulated glycolytic activity, resulting in increased pyruvate within the cell. It was reported, that in coronary artery disease macrophages, oversupply of pyruvate is connected with PD-L1 upregulation (Watanabe, Shirai et al. 2017). To check if a similar mechanism is causative for the promotion of PD-L1 protein expression during infection, cells were subjected to a high concentrations (12.5 mM) of pyruvate (Figure 5.21 [B]). Transport of macromolecules like pyruvate across plasma membranes is a tightly controlled process (Halestrap, Poole et al. 1990, Halestrap and Price 1999). To ensure uptake, pyruvate was actively transported into the cell via lipofection and relative mRNA levels of PD-L1 were measured 24 No altered expression could be observed, pointing towards other effects and strengthen the unique role of HK2. Regardless of the treatment method, cells subjected to pyruvate showed an altered phenotype pointing towards an increase in cell proliferation. As this could be beneficial in the course of an infection, cells were simultaneously treated with pyruvate and infected with *H. pylori*. Microscopic analysis showed that cells were less likely to die in the course of infection (Figure 5.21 [C]). To quantify this and amplify the effect, cell viability was assessed 48 h post infection via MTT assay (Figure 5.21 [D]). In the colorimetric assay, reduction degree of the yellow tetrazole MTT to purple formazan is measured spectrophotometrically. Reduction depends on activity of mitochondrial reductase, therefore providing a direct connection to metabolic activity, reflecting active cells and is therefore referred to as ‘cell viability’ (Alley, Scudiero et al. 1988, Bernas and Dobrucki 2002). Treatment with pyruvate does not influence non-infected cells but leads to a significant increase of cell viability in *H. pylori* infected condition.

To conclude, as a late response to *H. pylori* infection, elevated levels of pyruvate were observed. Addition of pyruvate failed to upregulate PD-L1, despite being a direct implication of the previously identified metabiotic upregulation (Figure 5.13 & Figure 5.14) which was shown to participate in PD-L1 regulation (Figure 5.15 [A]).

Under infection, pyruvate subjected cells were more resistant towards apoptosis, likely due to improved proliferation rate, highlighting the importance of the activation of the glycolytic pathway in the course of infection.

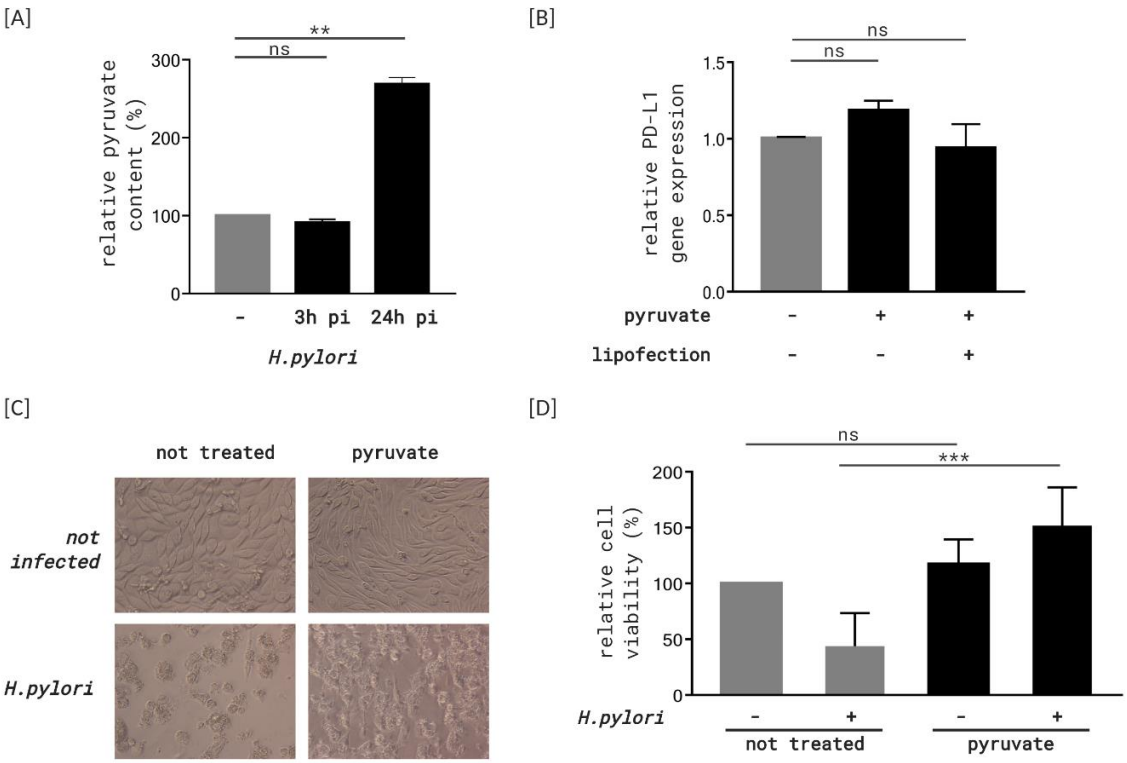


Figure 5.21 | Increased pyruvate content leads to more resilience towards *H. pylori*.
[A] AGS were infected for 3 h, 24 h with H. pylori, MOI50 or left not infected. Pyruvate content was measured mass via UPLC and normalized to uninfected sample. Pyruvate was measured by Robert Hurwitz. [B] AGS were extracellularly treated or transfected with 12.5 mM pyruvate or left untreated. Relative PD-L1 gene expression was assessed via qRT PCR in respect to the untreated condition. [C] Brightfield images of AGS, uninfected, infected with *H. pylori* MOI 50 for 24 h [D] Relative cell viability was determined using MTT assay

Chapter

6. Discussion

The gastric bacterium *H. pylori* is found in half of the world population. Infection with it elicits an acute inflammatory response which can become chronic and develop to atrophic gastritis, intestinal metaplasia, dysplasia and even progress towards gastric cancer (Correa, Haenszel et al. 1975) in 1 % of the infected people (Peek and Crabtree 2006). An essential role in inflammation and carcinogenesis plays the immune checkpoint inhibitor PD-L1 which is found aberrantly expressed in 30 to 50 % of gastric cancer biopsies (Böger, Behrens et al. 2016, Wang, Zhu et al. 2018, Liu, Choi et al. 2020). Hence, this thesis aimed to understand the pattern causing its upregulation to understand its role in bacterial colonization and gastric carcinogenesis.

Dissection of *in vitro* infection with *H. pylori* revealed two distinct upregulating incidents: An early, inflammation-driven activation pattern, triggered by the newly discovered PAMP ADP-heptose and a metabolic regulation, dependent on HK2 upon prolonged infection.

6.1. ADP heptose recognition by ALPK1 regulates acute PD-L1 response

The first upregulation phase is inflammation-related via a NF-κB signaling cascade. In the course of this study, the bacterial metabolite ADP heptose was identified as a fast and potent inflammation activating substance. The sugar activates the host kinase ALPK1 which was reported to be indispensable for the formation of TIFAsomes and the subsequent release of NF-κB to shuttle into the nucleus. In dependency of the cagT4SS, the ADP heptose / ALPK1 axis induces PD-L1 during early *H. pylori* infection.

6.1.1. PD-L1s link to inflammation

Early upregulation of PD-L1 correlates with induction of the chemokine IL-8 in response to *H. pylori*, indicating similar regulating mechanism. This is supported by evidence of a NF-κB binding site present withing the PD-L1 promoter region (Huang, Wen et al. 2013) and gastric cells shown to elicit gene expression of the ligand in response to stimulation with the NF-κB inducer TNFα (Wu, Lin et al.

2010). Further tests carried out with bacterial mutants indicated a dependency of the highly conserved bacterial gene island cagPAI. Encoded proteins are able to form a T4SS, reported to be indispensable for activation of NF-κB(Backert and Naumann 2010), underlining its role in NF-κB-mediated PD-L1 induction. Previously described effector molecules, translocated via the *H. pylori* secretion system are CagA (reviewed in (Hatakeyama 2014)), peptidoglycan (Viala, Chaput et al. 2004), chromosomally derived DNA (Varga, Shaffer et al. 2016) and the sugar HBP (Zimmermann, Pfannkuch et al. 2017). Bacterial mutants, unable to perform complete HBP synthesis, Δ*rfaE,* failed to induce PD-L1. *H. pylori* Δ*rfaE* possess an intact cagT4SS and were shown to successfully translocate CagA but abrogated the inflammatory response (Zimmermann, Pfannkuch et al. 2017). This indicates, that expression of PD-L1 is independent of CagA, which is in contrast to published data from *in vitro* experiments (Lina, Alzahrani et al. 2015, Holokai, Chakrabarti et al. 2019). However, within these studies, infection was performed for at least 24h, indicating that early ligand induction occurs independent of the late induction.

6.1.2. ADP heptose, the new PD-L1 inducer

A variety of pathways are capable of inducing proinflammatory signaling. For *H. pylori* at least sixteen different host cell signaling cascades and numerous bacterial factors were proposed to participate (reviewed in (Backert and Naumann 2010)). In recent years, another one was added, when Zimmermann and colleagues introduced heptose-1,7-bisphosphate (HBP) as an effector of the *H. pylori cag*T4SS, able to activate NF-κB (Zimmermann, Pfannkuch et al. 2017). The sugar HBP is the product of the second step in the ADP heptose synthesis pathway, in which D-sedoheptulose 7-phosphate is converted into ADP-D,D-heptose or ADP-L,D-heptose to be incorporated into the inner and outer core oligosaccharide of LPS, catalyzed by the glycosyltransferases RfaC and RfaF (Gronow, Brabetz et al. 2000, Kneidinger, Marolda et al. 2002). The outer core region differs highly among bacterial strains. By contrast, structural features of the inner core region are well conserved within a bacterial genus and are even shared by distantly related bacteria (Raetz and Whitfield 2002). In accordance was an innate immune role for heptose metabolites described, following infection with a

range of Gram-negative bacteria, including *Neisseria*, *Escherichia, Citrobacter* or *Shigella* (Gaudet, Sintsova et al. 2015, Gall, Gaudet et al. 2017, Stein, Faber et al. 2017, Carson, Barry et al. 2020). Central to the HBP-mediated inflammatory response is the ALK1-TIFA axis. Activated ALPK1 leads to TIFA threonine phosphorylation in position 9 (T9), which is recognized by a forkhead-associated domain (FHA) of adjacent TIFA molecules, resulting in agglomerations, called TIFAsomes, which contain additional proteins such as TRAF2. Subsequently, the TAB2/3-TAK1 complex is recruited, activating the IKK complex, which degrades the NF-kB inhibiting complex IκB, leading to nuclear p65 translocation (Zimmermann, Pfannkuch et al. 2017).

Results, demonstrating HBPs potential to cause innate immunity were acquired from experiments in which cells were either infected with bacterial mutants, unable to perform complete ADP heptose synthesis pathway such as Δ*rfaE*, or stimulated with synthesized HBP. However, mass spectrometry of *H. pylori* extracts revealed HBP amounts, insufficient to induce NF-κB activation as it is observed during infection. Screening for an inducer of early inflammatory responses led to the identification of ADP heptose, the pathway end product. Here 10-times higher concentration than HBPs were noted. It was speculated that not the RfaE-generated product HBP but rather ADP heptose is causative for the elicited immune response, as the bi-functional adenylyltransferase catalyzes two steps of the ADP heptose synthesis pathway. Besides the conversion of D-glycero-D-manno-heptose 1-phosphate into HBP by phosphorylation it adenylates D-glycero-D-manno-heptose-1 phosphate, generating ADP-D-glycero-D-manno-heptose (ADP-D,D-heptose) (Chang, Wang et al. 2011). ADP-D,D-heptose as well as its isomer ADP-L,D-heptose were able to trigger NF-κB signaling. In *H. pylori*, the inner core region consists of a hexa-saccharide (Kdo-LD-Hep-LD-Hep-DD-Hep-Gal-Glc) (Li, Liao et al. 2016). Given that both heptose isomers are present in the well conserved inner core region, immune stimulating ability is another validation that the bacterial metabolite acts as a PAMP.

LPS, whose core oligosaccharide is partially made up of heptose metabolites, was shown to induce PD-L1 expression in a TLR4-dependent manner in tumor

cells (Huang, Zhao et al. 2005). The gastric cancer cell line AGS was also reported to regulate this particular PRR in response to *H. pylori* infection (Su, Ceponis et al. 2003) and to regulate PD-L1 following *H. pylori* derived LPS stimulation (Li, Xia et al. 2018). However, experiments performed within this thesis showed contradictory results. Here, even LPS derived from *E.coli,* which exhibits 1 000-fold stronger biological capacity than *H. pylori*-derived LPS, did neither induce a proinflammatory response as measured by IL-8, nor PD-L1 induction. This is consistent with the finding that TLR4, which is the major receptor for LPS recognition, is present in AGS cells, but not responsive towards stimulation, resembling primary antral cells which lack expression of this PRR (Bäckhed, Rokbi et al. 2003).

Given that ADP heptose is sensed by ALPK1, cells deficient for the kinase were tested for their ability to induce PD-L1 gene expression. KO cells were able to trigger upregulation of the ligand 24h post infection but failed 3h post infection which shows the kinase-dependency at early stages of infection. In the course of *H. pylori* infection, gastric epithelial cells themselves are able to produce cytokines (Watanabe, Asano et al. 2010, Lin, Xu et al. 2015). Short treatment with TNFα and IFNβ resulted in PD-L1 upregulation, however irrespective of ALPK1, indicating that the observed early ligand regulation occurs independent of autocrine or paracrine cytokine-mediated signaling events as a kinase knockout was shown to abolish upregulation.

6.1.3. Comparing HBP with ADP heptose

Direct comparison of the previously identified PAMP HBP and ADP heptose showed activation by HBP requires a 100-fold higher concentration, marking it as a far less potent stimulus. Importantly, this observation is not contradictory to the previously published data as HBP has the ability to trigger NF-κB signaling. However, the required amount is not found in bacterial lysates, showing that the PAMP responsible for early immune activation is the monosaccharide ADP heptose. Indeed, crystal structure for ADP-Hep-bound ALPK1 has been determined, whereas prior to ALPK1 recognition, HBP needs to be converted into

ADP-heptose 7-phosphate by host adenylyltransferases as it was proposed for enzymes of the nicotinamide/nicotinic acid mononucleotide adenylyltransferase (NMNAT) family (Zhou, She et al. 2018). This could be beneficial for the immune system as it is capable of sensing a range of related bacterial metabolites, such as HBP with the same PRR ALPK1 by converting the metabolites into substances like ADP-heptose 7-phosphate, resembling the actual receptor ligand ADP heptose.

ADP heptose and liposomally-transported HBP were both able to activate NF-κB. However, only stimulation with the pathway end product resulted in induction of PD-L1. It could be hypothesized, that the low proinflammatory response to HBP is not sufficient and a certain NF-κB activation threshold needs to be passed. The discrepancy between the sugars could also be caused by the needed conversion of HBP into ADP-heptose 7-phosphate in order to trigger an inflammatory response which requires time and will accordingly delay the PD-L1 induction response (Zhou, She et al. 2018).

In contrast to HBP, ADP heptose is capable to activate an immune response when added extracellularly. The subsequent PD-L1 response appears to contradict the finding, that a functioning T4SS is required for signal transduction in *H. pylori*. Similarly, in *Y. pseudotuberculosis* the T3SS-stabilising protein YopB was found to be essential for ADP heptose dependent NF-κB activation (Zhou, She et al. 2018). Possible explanation include the presence of unspecific traffic or uptake of the sugar over the membrane, ADP heptose-specific transporters or a (transiently) membrane-associated ALPK1. For bacteria, such as *H. pylori* or *Y. pseudotuberculosis* the sugar appears to remain within the cell and reaches the host only after formation of a bacterial secretion system. Of note, in contrast to 30 min infection (Zimmermann, Pfannkuch et al. 2017), 3 h infection with *H. pylori* Δ*cagPAI* elicited inflammatory response (Suppl.Figure 9.2). Thus, it can be assumed that under physiological condition the sugar is kept within the periplasmic space and is only released following disruption of the bacterial membrane caused by formation of a secretion system or bacterial lysis.

6.1.4. Implications of ADP heptose/ALPK1

Within this thesis, it could be shown that in the course of *H. pylori* infection, the fast PD-L1 inducing determinant is the bacterial PAMP ADP heptose, which is sensed by the host ALPK1, activating NF-κB. The broad signaling consequences of ADP heptose challenged wildtype cells in comparison to cells deficient for ALPK1 had to be deciphered. The microarray tackling this challenge, revealed that *H. pylori* infected cells as well as ADP heptose challenged cells elicited a strong and similar immunogenic response, activating a range of inflammatory pathways. This effect was almost completely abolished in cells deficient for ALPK1. From the obtained results, it can be concluded that ADP heptose is exclusively sensed by the kinase and that the ADP heptose/ALPK1 initiated signaling is the major driver any response towards *H. pylori*, early in the infection process.

The described results shows that ADP heptose is one of the few bacterial metabolites which are able to act as a PAMP. It is found in a range of Gram-negative bacteria (Suppl.Figure 9.3) marking it as an ideal target for PRR recognition. Of note, in a few Gram-negative bacteria, including *Brucella* (Moreno, Pitt et al. 1979), *Legionella* (Knirel, Moll et al. 1996), *Francisella* (Okan and Kasper 2013) and *Moraxella* (Pokorny and Kosma 2016), ADP heptose is replaced by another sugar whereas some Gram-positive bacteria, like *Streptomyces fimbriatus* and *Streptomyces hygroscopicus* (Tang, Guo et al. 2018), are capable of synthesizing ADP heptose.

In an *in vivo* mouse model using ALPK1-deficient mice challenged with ADP heptose or the Gram-negative bacteria *Burkholderia cenocepacia* (Zhou, She et al. 2018), a phenotype reflecting the results obtained from the performed microarray within this thesis could be observed. ADP heptose challenging elicited a strong inflammatory response which was accompanied by robust neutrophil influx and completely abolished in the absence of ALPK1. In line with this, the bacterial load in the lung of mice was much higher in the absence of the kinase, showing the impact of an early inflammatory response on the colonization effectiveness of bacteria. Especially for *H. pylori* early phase of infection is crucial as a successful

colonization leads to lifelong bacterial persistency in the majority of people (Lambert, Lin et al. 1995), increasing the risk for gastric cancer development.

6.1.5. Outlook

The causative pathway for early PD-L1 induction could clearly be assigned to activation of the ADP heptose / ALPK1 signaling axis. Based on the obtained findings, the following experimental investigations are suggested.

To determine if early regulation of PD-L1 is exclusively dependent on inflammation or a consequence of multiple ADP heptose / ALPK1 downstream events, experiments targeting specifically NF-κB transcription can be performed. The promoter region of the ligand contains binding sites for several cytokine-induced transcription factors (Zerdes, Matikas et al. 2018). However, epithelial cells are only able to secrete a very limited range of cytokines. Therefore, it is expected, that NF-κB is the sole activator.

It appears to be a by-chance consequence of bacterial secretion system formation, that ADP heptose is transported into host cells. Investigations including Gram-negative bacteria with differing LPS structures and their ability to transport metabolites into the host can be studied to elucidate the process. Exemplary, within the core oligosaccharide of the Gram-negative bacterium *Legionella,* ADP heptose is substituted by the homomorphic molecule D-mannose (Knirel, Moll et al. 1996) and early *in vitro L pneumophia* infection led to, compared to *H. pylori*, a rather weak NF-κB activation, attributed to bacterial flagellin (Bartfeld, Engels et al. 2009). The second was found to be dependent of the bacterial secretion system Dot/Icm and replication (Bartfeld, Engels et al. 2009). It can be speculated that the late induction is triggered by a bacterial metablite, whose immunogenic activity was delayed caused by the need to convert mannose into a structure recognizable by ALPK1.

A broad range of pathways were shown to be upregulated in an ADP heptose / ALPK1-specific manner. Yet, many of these signaling cascades reflect NF-κB

activation and a clear dissection of independently upregulated genes remains to be accomplished. Usage of NF-κB-specific inhibitors during treatment with the LPS metabolite or direct comparison with other NF-κB inducers can be applied.

The finding, that the immune checkpoint receptor and PD-L1 binding partner PD-1 is downregulated in ALPK1 knockout cells fits the observation that ligand regulation is under the control of the PAMP ADP heptose, either by directly triggering NF-κB signaling or via increased inflammation. This could imply a role for ALPK1 beyond its PRR activity towards maintenance of cell homeostasis. Results, pointing to equivalent mechanism were obtained from ALPK1$^{-/-}$ mice, infected with the commensal pathobiontic bacterium *Helicobacter hepaticus* (Ryzhakov, West et al. 2018). Deficiency restricted the activation of Th1 by favoring Treg and Th17 responses, resulting, in the absence of IL-10 signaling, in exacerbated colitis. This is of importance as the PD-L1:PD-1 axis is known to balance Th1/Th2 and Treg/Th17 cell subtypes (Wu, Liu et al. 2019). Moreover, ALPK1$^{-/-}$ bone marrow-derived macrophages secreted in response to infection high levels of IL-12 (Ryzhakov, West et al. 2018), a cytokine negatively regulated by PD-1 (Ma, Ni et al. 2011). Taken together, it is of importance to directly elucidate the connection of PD-L1:PD-1 signaling and ALPK1 in the infection context with Gram-negative bacteria.

6.2. Isoform switch to HK2 promotes PD-L1 expression

Cells, deficient for ALPK1 displayed upregulation of the immune inhibitor ligand PD-L1 in the late course of *H. pylori* infection, pointing towards underlying mechanism independent of the ADP heptose/ALPK1 axis. Dissection of the inducing factor revealed ligand upregulation driven by a metabolic deregulation. The characteristic of the gastric bacterium to extract cholesterol, increased metabolic activity with specifically glycolytic activity within the host. The subsequent increase in the glycolysis pathway end product pyruvate was linked to improved host cell viability. Furthermore, the induced metabolic upregulation was followed by an expression shift of the glycolytic enzyme hexokinase. The initially favored HK isoform 1 was downregulated, whereas isoform 2 protein increased. A combination of knockdown and knockout experiments revealed a dependency of PD-L1 on the hexokinase shift.

6.2.1. PD-L1s link to cholesterol depletion

Several contradictory reports exist regarding virulence factors of *H. pylori* and their impact on PD-L1 induction. Consistent with other studies, it could be proven that upregulation of PD-L1 is independent of the virulence factor VacA (Das, Suarez et al. 2006). Contradictory reports regarding CagA and cagPAI's involvement exist (Das, Suarez et al. 2006, Lina, Alzahrani et al. 2015). In the here applied experiments, no significant contribution of these factors was observed, which can be attributed to bacterial strain differences or the used infection model. However, infection with *H. pylori Δcgt*, which are unable to glycosylate cholesterol and subsequently perform the process of cholesterol extraction from host membranes, caused significantly lower amounts of PD-L1 24 h post infection compared to wildtype bacteria. *H. pylori* are auxotroph for cholesterol and need to either bind free cholesterol found in the medium or extract sterols present in host cell lipids, to which it is chemoattracted (Hirai, Haque et al. 1995, Wunder, Churin et al. 2006).

Supported is the hypothesis that PD-L1 gets regulated in a cholesterol dependent manner by *in vivo* experiments with mice on a high-cholesterol diet. These mice, when infected with *H. pylori*, showed reduced bacterial load compared to mice on

a regular diet. The effect was dismissed in Rag1$^{-/-}$ mice (lacking T and B cells) (Wunder, Churin et al. 2006). This means, applied to the hypothesis, that under high cholesterol diet, the host metabolism would not be affected, which would avoid the expression of PD-L1 and the subsequent inhibition of immune cells. Similarly, in wildtype mice infected with *H. pylori* Δ*cgt*, bacterial clearance occurred already 48 h post infection. They further showed that CGT activity triggers avoidance of T cell response (Wunder, Churin et al. 2006), pointing towards the involvement of immune cell inhibitory molecules.

6.2.2. Metabolic reprogramming

Comparison of microarray data, obtained from cells which were 24h infected with wildtype or Δ*cgt* mutants revealed that a large number of genes are differentially expressed only in response to the sterol extracting activity. In accordance with the fact that cholesterol depleted cells are in need for sterol replacement, a large subset of deregulated genes are involved in metabolic processes. Glycolysis, the first of the main metabolic pathways of cellular respiration, through which one molecule glucose is metabolized into two molecules of pyruvate and energy is stored in the form of ATP and NADH (reviewed in (Alberts 2014, Alfarouk, Verduzco et al. 2014)), was found to be upregulated in host cells upon bacterial challenge. It can be reasoned, that the cause of the increase is the demand for the end product pyruvate which gets converted into acetyl-coA, the starting product for the mevalonate pathway, which is required for cholesterol biosynthesis (Miziorko 2011).

To test, which effect accumulation of pyruvate can exert, cells were challenged with the monocarboxylate, which resulted in significant increase in cell viability of infected cells compared to not-pyruvate-treated conditions. A comparable observation was made in uninfected breast cancer cells following pyruvate treatment, which was assigned to an increased proliferation as a consequence of an accelerated mitochondrial activity (Diers, Broniowska et al. 2012). In the context of *H. pylori* infection, it can be assumed, that it serves two purposes: (1) precursor

for cholesterol replenishment and (2) increase in cellular proliferation to compensate for cellular damage caused by the pathogen.

6.2.3. HK2, a new PD-L1 inducer

Within this thesis, it could be shown that immune inhibitor ligand PD-L1 upregulation is a consequence of enhanced metabolic activity caused by cholesterol depletion. qRT-PCR studies of infected cells revealed an expression shift from HK1 to HK2 in the course of infection. The enzyme hexokinase catalyzes the second step of the glycolysis by phosphorylating glucose to glucose-6-phosphate and thereby trapping it within the cell, as only the unphosphorylated sugar can shuttle in and out of the cell via GLUT receptors (reviewed in (Alberts 2014)). Expression of HK isoform 1 is ubiquitously found but HK2 activity is restricted to adipose and skeletal tissue and the heart (Roberts and Miyamoto 2015). The shift from HK1 towards HK2 expression is of especial importance, as such alterations were shown to initiate tumorigenesis, e.g. in human liver samples, where the low-affinity HK4 is the predominant isoform but a switch towards HK2 was noted during hepatocarcinogenesis (Guzman, Chennuri et al. 2015). It could be speculated that the observed upregulation of HK2 occurs as consequence of the used infection cell model AGS, a gastric cancer cell line and as expression of the hexokinase isoform was found in many cancers, including pancreatic and ovarian cancer (Anderson, Marayati et al. 2016, Siu, Jiang et al. 2019). However, it could be shown that also in human gastric primary cells, *H. pylori* infection leads to an upregulation of HK2 in a CGT-dependent manner. It has to be taken into consideration that human samples originate from gastric sleeve resections. HK2 is insulin-sensitive and obesity of the patients could have changed its expression level. However, no such link is known to date.

In macrophages from patients with coronary artery disease, treatment with 2-deoxy glucose, an agonist of HK isoform 1-3, showed decrease in the previously noted aberrant expression of PD-L1 (Watanabe, Shirai et al. 2017). By using siRNA-mediated knockdowns, induction dependency could be narrowed down to HK2 in infected gastric cells. As gastric cancer and PD-L1 expression are connected, the correlation of HK2 expression should be investigated too. For ovarian cancer,

expression was reported to be linked with cancer cell migration as well as invasion, and in KRas-driven lung cancer, and ErbB2-driven breast cancer mouse models, HK2 was proven to be crucial for tumor initiation and maintenance (Patra, Wang et al. 2013, Siu, Jiang et al. 2019).

An altered metabolism distinguishes cancer cells from their healthy counterparts. Therefore, many studies provide rationales for targeting glycolytic enzymes for cancer therapy. The herewith presented data, that HK2 upregulates the immune checkpoint inhibitor PD-L1, provides additional reasons to target these enzymes. However, concerns arose in respect to potential adverse impacts affecting the glycolysis in healthy cells under normal metabolic homeostasis. Apart from cardiac, skeletal or adipose tissue, targeting HK2 for this purpose should be considered as safe and indeed, for lung cancer, inhibition of HK2 was shown to slow down cancer cell growth and induce apoptosis as well as autophagy *in vitro* and *in vivo* (Wang, Wang et al. 2016). Knowledge concerning hexokinase and gastric cancer is limited. Yet, the two studies, investigating human gastric carcinomas, reported aberrant HK2 expression in 16-21% of the cases which was linked to worse prognosis (Rho, Kim et al. 2007, Qiu, Han et al. 2011). In contrast, aberrant PD-L1 expression in gastric adenocarcinomas occurred in 30-50 % of the tested patients (Böger, Behrens et al. 2016, Wang, Zhu et al. 2018, Liu, Choi et al. 2020). It is likely, that apart from metabolic reprogramming, other factors contribute to deregulation of the ligand in chronic *H. pylori* infection, as a meta-analysis described correlations with Epstein-Barr virus infection as well as microsatellite instability (Gu, Chen et al. 2017).

6.2.4. Concepts of HK2 activity

By using CRISPR/Cas9 modified cells, it could be further confirmed that *H. pylori* specifically upregulated PD-L1 in a HK2-dependent manner. The observation that the glycolytic enzyme HK2 is associated with immune checkpoint PD-L1 regulation is a novel finding, linking metabolism and immunity. However, the exact underling regulatory pathway remains elusive and requires further attention. From the performed experiments, it can be concluded that the direct effect of HK activity,

production of pyruvate, did not affect ligand induction. This suggests, that another mechanism is involved. A possibility is by moonlighting activity, the ability of proteins to perform multiple autonomous and mechanistically different functions (Huberts and van der Klei 2010). Out of the ten proteins involved in glycolysis, for seven, a moonlighting activity has been shown (Kim and Dang 2005, Sriram, Martinez et al. 2005). HK2 specific target sequences were found within the promoter region of glucokinase and hexokinase 1 in *Saccharomyces cerevisiae*, regulating their activity (Rodríguez, De La Cera et al. 2001).

Of note, an innate immune receptor function for hexokinase has been shown, by which HK2 detects bacterial N-acetylglucosamines, a sugar subunit of the backbone of peptidoglycans, from *Staphylococcus aureus*, resulting in enzyme dissociation from the mitochondria and thereby activating the NLRP3 inflammasome (Wolf, Reyes et al. 2016). Shift and upregulation of hexokinase isoform 2 could lead to detection of *H. pylori* components, thereby activating the inflammasome and subsequently PD-L1 through IL-1β signaling. This theory is supported by the observation that PD-L1 upregulation in chronic *H. pylori* infection was reported to depend on the presence of peptidoglycans (Lina, Alzahrani et al. 2015).

6.2.5. Outlook

The causative pathway for late PD-L1 response were identified to be mediated by cholesterol depletion-driven expression shift from the glycolytic enzyme HK1 to HK2. Based on the obtained results, the following next steps are suggested:

Unpublished data from the department provided evidence that key enzymes for the mevalonate pathway are upregulated in dependency of CGT activity. Additional experiments can be performed to clarify if the observed expression shift towards HK2 is necessary for activation or activity of the cholesterol-replacing pathway. According to this theory, mice, harboring a conditional HK2-knockout should be more susceptible to tissue damage following *H. pylori* infection, as host cell membranous cholesterol will less efficiently be replaced.

The signaling mechanism, downstream of HK2 which mediates PD-L1 regulation can be further explored. A possible experiment to check for moonlighting activity is by investigating the nucleus-shuttling ability upon infection by ChIP-sequencing and the subsequent validation with cells, expressing truncated HK2 variants. Binding ability to possible targets like PD-L1 should be studied. Most of the proteins, identified to harbor moonlighting ability, are the highly conserved enzymes of the sugar metabolism pathways (reviewed in (Huberts and van der Klei 2010)). However, it has to be taken into account, that inter-species sequence similarity of a given moonlighting enzyme is not connected with the ability to moonlight in both species (Ozimek, Kötter et al. 2006). Therefore, the benefit of any mouse experiments will be rather questionable.

To prove that HK2 is able to act as sensor for bacterial components, metabolites of bacterial origin should be tested, especially species known to interfere with host metabolic activity. Following infection with the obligate intracellular bacterium *Chlamydia* trachomatis, which is known to dependent on a variety of host-derived metabolic precursors and cofactors (reviewed in(Omsland, Sixt et al. 2014)), we could show, that HK2 and its mitochondrial association is increased (Al-Zeer, Xavier et al. 2017). However, if *Chlamydia*-derived metabolites are involved in this process needs to be elucidated.

In vivo data of infected mice revealed a connection between *H. pyloris* cholesterol extracting activity and a triggered avoidance of T cells (Wunder, Churin et al. 2006), pointing towards the involvement of immune checkpoint inhibitors. To validate this hypothesis, expression of the immune cell regulators PD-L1 and its binding partners PD-1, and B7-H1 should be evaluated in *in vivo* infection models. Common to *Helicobacter spp* is the presence of cholesterol within their membrane (Haque, Hirai et al. 1995). Likewise, it was shown for several other bacteria, including *Ehrlichia, Anaplasma* (Lin and Rikihisa 2003), *Mycoplasma* (Smith 1971), *Brachyspira* (Trott, Alt et al. 2001), and *Borrelia* (Ben-Menachem, Kubler-Kielb et al. 2003). Emphasis should be put on infection models including them. Of note, connections to PD-L1 increase were already published, for infection *Mycoplasma* (Goto, Konnai et al. 2017) or *Anaplasma*, were expression of the

ligand was linked with persistency by T cell exhaustion (Okagawa, Konnai et al. 2016). Given that within this thesis, for the first time a link was drawn between extraction of host-membranous cholesterol depletion, the potential role of sterol-extracting capacity of these bacteria in upregulation of the immune cell inhibitory ligand needs to be defined.

Chapter

7. Conclusion

Within the present work it could be demonstrated that induction of PD-L1 occurs as consequence of two distinct mechanism (Figure 7.1). For the first upregulating event, a new, very potent immune stimulus ADP heptose could be introduced which enables the cell to immediately react to invading Gram-negative pathogens. This high inflammatory response is accompanied by induction of the immune regulatory molecule PD-L1, most likely to balance this reaction. Cells which are deficient for the sugar sensor ALPK1 are basically blind to bacterial invasion, highlighting the role of the ADP heptose/ALPK1 signaling axis in initial bacterial recognition upon infection. In contrast, the late PD-L1 induction phase could be delignated to the glycolytic enzyme HK2. For the first time, cholesterol depletion activity of *H. pylori* was linked with metabolic deregulation and expression shift of hexokinase isoform 1 to 2. Furthermore, it can be concluded that an upregulated metabolism provides cellular advantage to withstand harmful damage by pathogens.

A general question to ask is if expression of PD-L1 and the subsequently triggered immune cell inhibition is beneficial or detrimental to the host in the course of an infection. It can be hypothesized that the inflammation-driven first induction phase is rather useful to prevent tissue damage following excessive immune response. This is supported as infection of mice, which are deficient for ALPK1 showed an exacerbated inflammatory response (Ryzhakov, West et al. 2018). The second induction peak of PD-L1 -on the other hand- follows not a heightened immunity but results from a deregulated host metabolism due to bacterial activity. This means that colonizing *H. pylori*, being auxotroph for cholesterol, are constantly extracting sterols from host membranes, thereby inhibiting immune cell activity via PD-L1 upregulation. Subsequently forming a micro niche in which they are protected from host immunity consequences. In accordance, *in vivo* expression of the ligand following *H. pylori* infection was connected with upregulation of a T cell subset, crucial for immunological tolerance towards the bacterium (Beswick, Pinchuk et al. 2007), thereby promoting bacterial persistency which increases the risk of a pathological outcome.

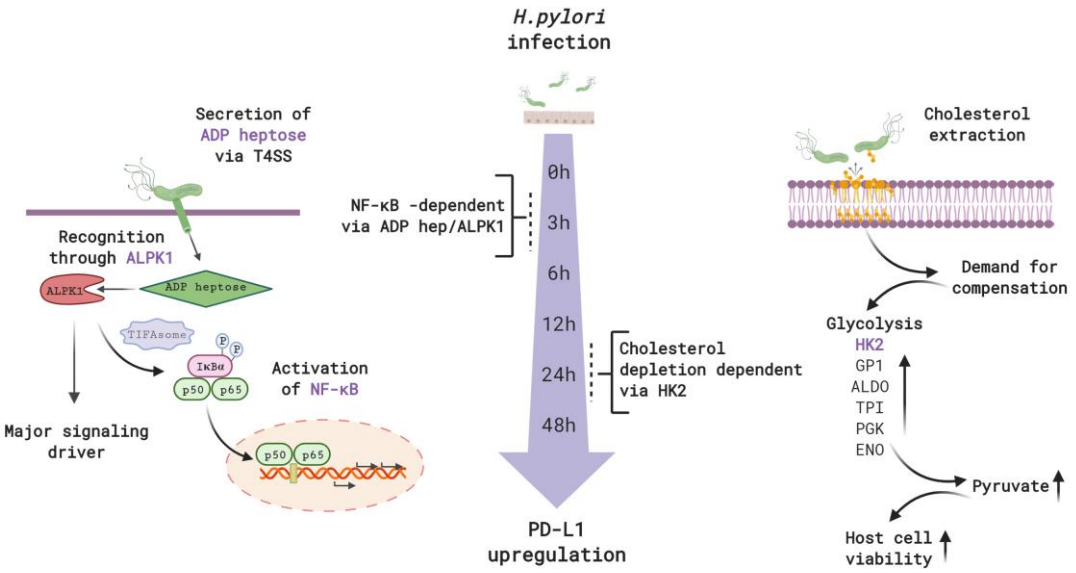


Figure 7.1 | Working model for PD-L1 regulation upon *H. pylori* infection
[A] When the Gram-negative bacterium *H. pylori* successfully invades the human body and reaches the epithelial lining, it forms a pore-like structure, the cagT4SS through which it injects bacterial factors into the cell. Among them is the LPS intermediate ADP heptose, which gets sensed by the host kinase ALPK1. Activated ALPK1 phosphorylates the protein TIFA, resulting in protein agglomerates called TIFAsomes. From this, a signaling cascade starts, leading to activation of the transcription factor NF-κB and the subsequent induction of PD-L1.
[B] The cholesterol-auxotroph bacterium exhibits chemoattraction towards regions of high cholesterol content in the host lipid membrane, called lipid rafts. Extraction of the sterol leads to a metabolic imbalance of the host, upregulating energy-gaining processes, like glycolysis. To compensate for the missing membrane compartment, pyruvate gets metabolized to acetyl-CoA to be used in the mevalonate pathway which results in the synthesis of cholesterol and the membranous reconstitution. During this process a switch of hexokinases from isoform 1 to 2 occurs which results in upregulation of the immune checkpoint ligand PD-L1. Furthermore, an increase in pyruvate production is noted, which increases host viability against the infection.

Chapter

8. References

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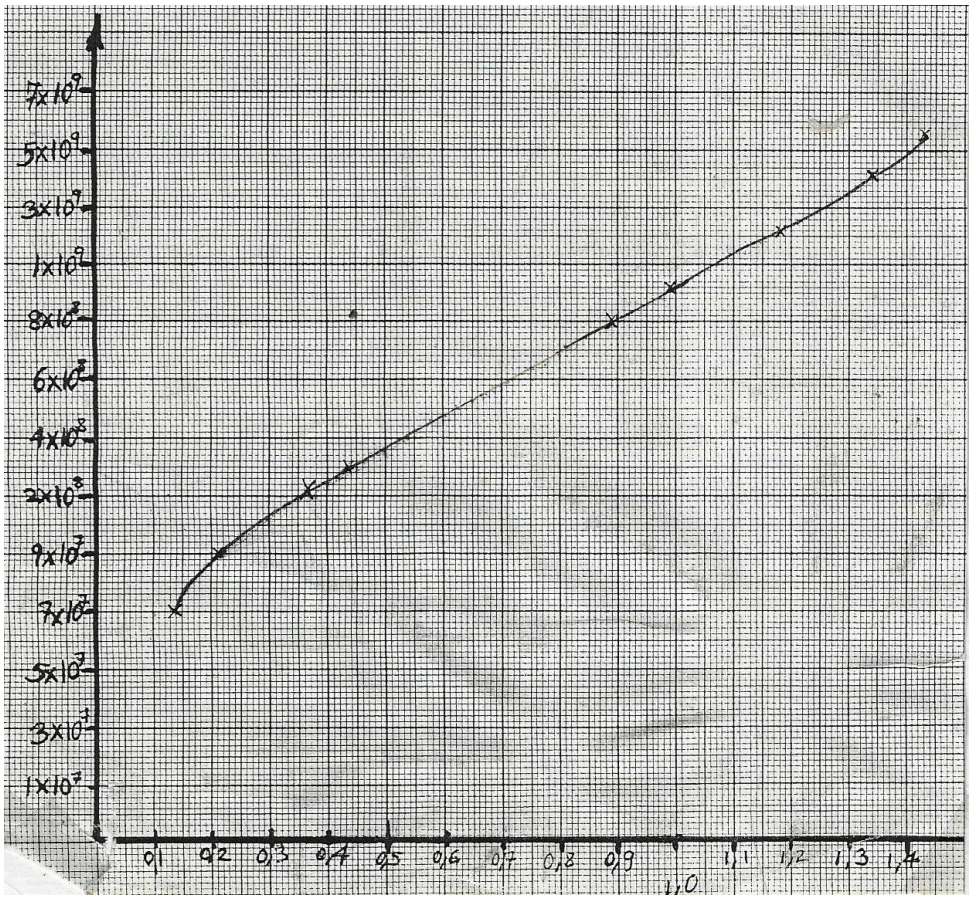
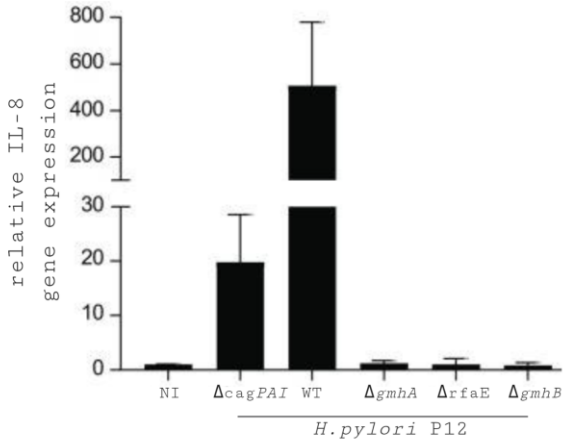
Chapter

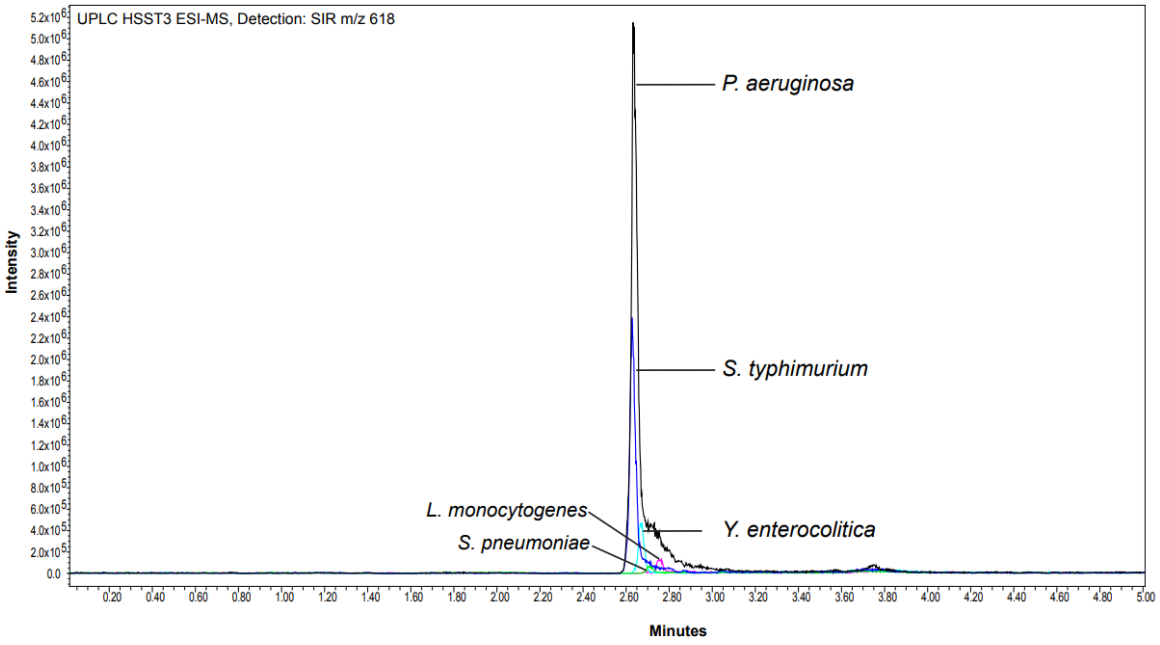
9. Appendix



Suppl.Figure 9.1 | Standard curve *H.pylori*
Graph of the OD_{550} values (x-axis) against the CFU/ml (y-axis) of *H. pylori*. OD_{550} of a serial, pre-defined bacterial dilutions was assessed. Bacteria were subsequently plated and number of CFU was reviewed three days later (performed by a Kirstin Hoffmann).



Suppl.Figure 9.2 | NF-κB activity of *H. pylori* Δ*cagPAI*
qRT PCR result for AGS cells, infected with MOI 100 of *H. pylori* WT and indicated mutants for 3 h and analyzed for IL-8 induction. Data shows relative induction compared with uninfected control. Data represent means ± SEM of 2 independent replicates.



Suppl.Figure 9.3 | ADP-heptose is present in lysates of other Gram-negative bacteria
Solid phase extracts from lysates of *Pseudomonas aeruginosa*, *Yersinia enterocolitica*, *Salmonella typhimurium*, *Streptococcus pneumonia* and *Listeria monocytogenes* were run over a Waters HSS T3 reversed phase UPLC. Shown are the chromatograms in SIR (Single Ion Recording) mode for m/z 618 (negative mode).

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Final Note

"There's more to see than can ever be seen

More to do than can ever be done

There's far too much to take in here

More to find than can ever be found"

(Circle of life)