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THE EXPRESSION OF HLA-B27 MODULATES INTRACELLULAR SIGNALING IN HUMAN MONOCYTIC MACROPHAGES

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You can never cross the ocean until you have the courage to lose sight of the shore.

Christopher Columbus

ABSTRACT

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The expression of HLA-B27 modulates intracellular signaling in human monocytic macrophages

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Reactive arthritis (ReA) is an inflammatory joint disease triggered by certain bacterial infections e.g. gastroenteritis caused by *Salmonella*. ReA is strongly associated to HLA-B27. However, the mechanism behind this association is unknown but it is suggested that the bacteria or bacterial compartments persist in the body. In this study, it was investigated whether the intracellular signaling is altered in HLA-B27-transfected U937 monocytic macrophages. Moreover, the contribution of HLA–B27 heavy chain (HC) misfolding was of interest.

The study revealed that p38 activity plays a crucial role in controlling intracellular *Salmonella* Enteritidis in U937 cells. The replication of intracellular bacteria was dependent on p38 kinase and the activity of p38 was dysregulated in HLA-B27-transfected cells expressing misfolding heavy chains (HCs). Also the double-stranded RNA -dependent kinase (PKR) that modifies p38 signaling was overexpressed and hypophosphorylated upon infection and lipopolysaccharide stimulation. The expression of CCAAT enhancer binding protein beta (C/EBPβ) was found to be increased after infection and stimulation. Increased amount of full length human antigen R (HuR), disturbed HuR cleavage and reduced dependence on PKR after infection were observed. All the findings were linked to HLA-B27 HCs containing misfolding-associated glutamic acid 45 (Glu45) at the peptide binding groove.

The results indicate that the expression of HLA-B27 modulates the intracellular environment of U937 monocytic macrophages by altering signaling. This phenomenon is at least partially associated to the HLA-B27 misfolding. These observations offer a novel explanation how HLA-B27 may modulate inflammatory response induced by ReA-triggering bacteria.

Keywords: reactive arthritis, HLA-B27, intracellular signaling, protein misfolding

TIIVISTELMÄ

ANNA SAHLBERG

HLA-B27-antigeenin ilmeneminen vaikuttaa solunsisäiseen signaalinvälitykseen ihmisen monosyytti-makrofagisoluissa

Lääketieteellinen mikrobiologia ja immunologia, Turun yliopisto, Turku ja Tartuntatautiseurannan ja -torjunnan osasto, Terveyden ja hyvinvoinnin laitos, Turku

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HLA - B27 on reaktiivisen niveltulehduksen (ReA) riskitekijä. Ei kuitenkaan tiedetä, mikä mekanismi on kasvaneen tautialttiuden taustalla. Yleensä ReA:n laukaisevat tietyt bakteeri-infektiot, kuten *Salmonellan* aiheuttama gastroenteriitti ja onkin todettu, että bakteeri tai sen osat voivat jäädä elimistöön pitkäksi aikaa. Tämän tutkimuksen tarkoituksena oli selvittää, onko solunsisäinen signalointi muuttunut HLA-B27–transfektoiduissa U937-linjan monosyytti-makrofageissa mahdollistaen *Salmonella* Enteritidiksen säilymisen soluissa. Lisäksi tutkittiin, onko HLA-B27-molekyylin raskasketjun (HC) epätäydellinen laskostuminen merkittävä tekijä havaittavissa muutoksissa.

Tutkimus osoitti, että solunsisäinen *S.* Enteritidiksen replikaatio on riippuvainen p38 kinaasin aktiivisuudesta. HLA-B27 transfektoiduissa soluissa, joissa HLA-B27-molekyylin raskasketjut sisältävät glutamiinihapon kohdassa 45, p38 aktiivisuus vaikuttaa olevan poikkeavasti säädelty verrattuna soluihin, joissa ei HLA-B27-molekyyliä ole. Tutkimuksessa tutkittiin myös kaksijuosteisesta RNA:sta riippuvaista kinaasia (PKR), joka säätelee *Salmonella*-infektion stimuloimaa p38 signalointia. PKR:n havaittiin yliekspressoituvan ja sen fosforylaatio todettiin heikommaksi kuin HLA-B27:ää ilmentämättömissä soluissa infektion ja lipopolysakkaridistimulaation jälkeen. Lisäksi CCAAT:hen sitoutuvan proteiinin (C/EBPβ) ilmeneminen on tällöin lisääntynyt. HLA-B27-transfektoiduissa soluissa C/EBPβ-tuotannon riippuvuus PKR:stä lipoplysakkaridistimulaation jälkeen on häiriintynyt. Havaitut muutokset liittyvät HLA-B27-molekyylin raskasketjun glutamiinihappo 45:een. Humaani antigeeni R (HuR) säätelee tulehdusreaktiota. Havaittiin, että täyspitkän HuR:n lisääntynyt määrä, häiriintynyt pilkkoutuminen ja vähäisempi riippuvuus PKR:stä infektion jälkeen korreloivat glutamiinihappo 45:n kanssa. Tulokset osoittavat, että HLA -B27 säätelee monosyytti-makrofagien solunsisäistä ympäristöä muuttamalla signalointia. Tämä ilmiö on ainakin osittain riippuvainen HLA-B27-raskasketjujen taipumuksesta laskostua väärin. Havainnot tarjoavat selityksen sille kuinka HLA-B27 voi säädellä ReA:n aiheuttajabakteerien indusoimaa tulehdusreaktiota.

Avainsanat: reaktiivinen niveltulehdus, HLA-B27, solunsisäinen signaalinvälitys, proteiinin laskostumisvirhe

TABLE OF CONTENTS

ABBREVIATIONS

AP-1	activator protein-1
ASK	apoptosis signal-regulating kinase
ATF	activating transcription factor
β2m	β2-microglobulin
BiP	binding immunoglobulin protein
CD	cluster of differentiation
C/EBPβ	CCAAT enhancer binding protein beta
CHOP	C/EBP-homologous protein
CNX	calnexin
CP	cleavage product
CRT	calreticulin
DNA	deoxyribonucleic acid
DRB	dsRNA binding
ds	double-stranded
eIF2α	eukaryotic translation initiation factor 2α
EOR	ER overload response
EPK	eukaryotic protein kinase
ER	endoplasmic reticulum
ERAD	ER-associated degradation
ERAP1	ER aminopeptidase 1
ERK	extracellular signal–regulated kinase
ERp57	ER stress protein 57
HBSS	Hank's balanced salt solution
HC	heavy chain
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HuR	human RNA binding protein
IFN	interferon
IL	interleukin
IRAK	IL-1 receptor-associated kinase
IRE1	inositol requiring enzyme 1
JNK	c-Jun NH2-terminal kinase
LAP	liver-enriched activator protein, C/EBPβ isoform
LIP	liver-enriched inhibitory protein, small C/EBPβ isoform
LPS	lipopolysaccharide
MAPK	mitogen activated protein kinase
MAPKAPK	MAPK activated protein kinase
MHC	major histocompatibility complex
mRNA	messenger RNA
MyD88	myeloid differentiation factor-88
NES	nuclear export sequence
NF-κB	nuclear factor kappa B

NO	nitric oxide
PBMC	peripheral blood monocytic/mononuclear cell
PBS	phosphate-buffered saline
PERK	PKR-like ER kinase
PKR	dsRNA -dependent kinase
PLC	peptide loading complex
PMA	phorbol myristate acetate
RA	rheumatoid arthritis
ReA	reactive arthritis
RNA	ribonucleic acid
ROS	reactive oxygen species
RT	room temperature
SCV	Salmonella containing vacuole
SF	synovial fluid
siRNA	small interfering RNA
SpA	spondyloarthropathy
SPI	Salmonella pathogenity island
STAT	signal transducer and activator of transcription
TAP	transporter associated with antigen processing
TIR	toll-interleukin 1 receptor
TLR	toll-like receptor
TNF	tumor necrosis factor
TRAF	TNF receptor-associated factor
U937	human monocytic cell line
UPR	unfolded protein response
UTR	untranslated region

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by Roman numerals I-III:

I. **Sahlberg A.S.***, Penttinen M.A.*, Heiskanen K.M., Colbert R.A., Sistonen L., Granfors K. Evidence that the p38 MAP Kinase Pathway is Dysregulated in HLA-B27-Expressing Human Monocytic Cells: Correlation with HLA-B27 Misfolding. Arthritis Rheum. 2007; 56: 2652-62.

II. **Sahlberg A.S.**, Ruuska M., Colbert R.A., Granfors K., Penttinen M.A. Altered PKR signalling and prolonged C/EBPβ expression is associated with HLA-B27 misfolding in monocytic cells. Scand J Immunol. 2012; 75: 184-192.

III. **Sahlberg A.S.**, Ruuska M., Granfors K., Penttinen M.A. Altered regulation of ELAVL1/HuR in HLA-B27–expressing U937 monocytic cells. PLoS One. 2013; 22; 8(7):e70377.

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1 INTRODUCTION

Reactive arthritis (ReA) belongs to a disease family of spondyloarthropathies (SpAs). It is an inflammatory joint disease triggered by infection elsewhere in the body. Often the triggering infection is gastroenteritis caused by *Campylobacteriae*, *Salmonellae*, *Shigellae* or *Yersiniae*, or respiratory or urinary tract infections caused by *Chlamydiae*. Human leukocyte antigen HLA-B27 is a well-known risk factor for ReA but it is still unclear how it contributes to the disease susceptibility or outcome. Patients with *Salmonella*-triggered ReA have prolonged production of *Salmonella*-specific antibodies. Moreover, it is clear that bacteria or bacterial compartments like lipopolysaccharides (LPS) and nucleic acids are able to persist in the body for a long period. It has been suggested that the host-bacteria interaction is disturbed but it is not known by which mechanism HLA-B27 is involved in the disease pathogenesis.

Previous studies by our group indicate that HLA-B27 expressing cells are not able to eliminate intracellular *Salmonella* and moreover, *Salmonella* Enteritidis is able to replicate within the cells. HLA-B27 molecule has a tendency to misfold and form homodimers. These uncommon features seem to be of importance since it has been shown that *Salmonella* replication is linked to the amino acid composition of HLA-B27 heavy chain (HC). When glutamic acid at position 45 at the HLA-B27 peptide binding groove is replaced, HLA-B27 HCs are folded more efficiently and the intracellular bacteria are eliminated.

Intracellular signaling molecules are important mediators of the immune response. For example proinflammatory cytokine tumor necrosis factor alpha (TNFα) secretion is upregulated during infection. Anti-TNFα therapy is effective in the treatment of SpA but it might not completely halt the disease progress. Therefore the pathogenesis of ReA and all SpAs need to be understood in order to find better treatment and manage the disease. It can be suggested that the expression of HLA-B27 affects intracellular signaling in macrophages. The question how HLA-B27 modulates the intracellular environment in favor of the bacteria is of importance in understanding of ReA pathogenesis.

In this study it was investigated whether the HLA-B27 expression alone modulates the intracellular environment of monocytic macrophages enabling the persistence of bacterial compartments and also explaining the abnormal immune response. The study was designed to clarify whether there are signaling pathways modulated in the presence of HLA-B27 that can be linked to the prolonged intracellular survival of *S.* Enteritidis. Moreover, it was investigated whether the abnormal misfolding of HLA-B27 HCs is involved in the HLA-B27 modulatory effects.

2 REVIEW OF THE LITERATURE

2.1 Spondyloarthropathies

Spondyloarthropathies (SpAs, also spondyloarthritides) are a group of inflammatory rheumatic diseases (Table 1.). Although the pathogenesis of these diseases differ from each other, they share common clinical and etiological features (Table 2.). SpAs are characterized by inflammation of the spine and at tendon or ligament attachment sites (Reviewed in Benjamin and McGonagle, 2009). SpAs also have a tendency towards familial aggregation (Calin et al., 1984) and they share genetic features including an association to HLA-B27. HLA-B27 was linked to ankylosing spondylitis (AS) already in 1973 (Brewerton et al., 1973). Also the susceptibility to ReA was associated to HLA-B27 (Aho et al., 1973). The mechanism by which HLA-B27 contributes to disease susceptibility and severity is poorly understood and in fact, the pathogeneses of SpAs are still unclear. However, it is known that bacterial and mechanical stresses are of importance in the development of SpAs (Reviewed in Ambarus et al., 2012, Girschick et al., 2008).

Table 1. Spondyloarthropathies (SpAs)

Condition
Reactive arthritis (ReA)
Ankylosing spondylitis (AS)
Psoriatic arthritis (PsA)
Arthritis associated with ulcerative colitis / Crohn's disease (spondylitis associated with inflammatory bowel disease, SpAIBD)
Undifferentiated spondyloarthropathy (USpA)
Juvenile spondyloarthropathy (JSpA)*
Acute anterior uveitis*

Modified from Carter, 2010, Stolwijk et al., 2012
*Classified as SpA according to Assessment of SpondyloArthritis international Society (ASAS) (Zeidler et al., 2011)

2.2 Reactive arthritis

ReA is an infection-induced systemic illness. The term ReA was introduced in 1969 and described as an aseptic polyarthritis during or after infection elsewhere in the body (Ahvonen et al., 1969). Although no universal, validated classification for ReA is available at present (Reviewed in Stolwijk et al., 2012), ReA is typically described as an asymmetrical oligoarthritis, often affecting large joints of the lower limbs. Also arthritis in the upper limbs and enthesitis (inflammation of the tendinous and ligamentous insertions) are common in ReA. Extra-articular features include inflammatory low back pain, conjunctivitis or uveitis (inflammation of the eye), and skin changes. ReA shares several common features with other SpAs such as asymmetrical oligoarthritis, inflammatory backache, Achilles tendonitis or plantar fasciitis, sacroilitis and a strong

association with HLA-B27. Symptoms typically develop within 1–4 weeks of the preceding infection caused by a microbe (Reviewed in Carter, 2006). In most cases, ReA is an acute disease and the duration of acute ReA usually varies between 3 and 5 months (Reviewed in Hannu et al., 2006) but it can be influenced by medical treatment. The triggering bacterial infection can be treated with antibiotics. Non-steroidal anti-inflammatory drugs (NSAIDs) as well as glucocorticoids are used to treat arthritis symptoms. However, up to 30-60% of ReA patients may develop a chronic and possibly severe disease (Reviewed in Carter, 2006 and, Rihl et al., 2006). Disease modifying drugs (DMARDs) like sulphasalazine are indicated for persisting symptoms. TNFα-blockers are effective in the treatment of different SpAs. However, in ReA, the possible persistence of the triggering bacteria must be taken into account since susceptibility to infections is increased during treatment with TNFα-blockers.

ReA diagnosis is usually a combination of typical clinical picture and the identification of the triggering infection (Reviewed in Hannu, 2011). As there is no diagnostic test or even established criteria for ReA diagnosis, the diagnosis is based on clinical characteristics and is a subject to clinical opinion (Reviewed in Ajene et al., 2013). Thus it is difficult to determine the exact incidence of ReA and most studies are conducted after outbreak of ReA triggering infections. The ReA incidence varies widely between different populations; the annual incidence is 6–27 cases per 100 000 infections (Reviewed in Hannu, 2011). Moreover, a recent meta-analysis combining 16 cohort studies suggests that the mean incidence of ReA is 12 cases per 1 000 *Salmonella* infections (Reviewed in Ajene et al., 2013). It can be stated that the complexity and discordance of ReA diagnosis leads to a variation in reported incidences. Moreover, often patients with typical picture of ReA are not diagnosed with preceding infection and can thus be labelled as undifferentiated spondyloarthropathy (uSpA) according to the European Spondyloarthropathy Study Group (ESSG) criteria (Reviewed in Misra, 2008). Epidemiological data also suggests that especially Chlamydia-induced ReA is underdiagnosed and asymptomatic chlamydial infections might be a common cause of ReA (Reviewed in Carter and Inman, 2011). Despite the controversy on ReA incidence, it is certain that ReA affects a significant number of individuals worldwide, especially in developing countries where the rate of food- and water-borne infections is high (Reviewed in Ajene et al., 2013).

Table 2. Characteristics of spondyloarthropathies (SpAs)

Clinical features
Sacroilitis with inflammatory back pain
Peripheral arthropathy
Absence of rheumatoid factor and subcutaneous nodules
Enthesitis
Extra-articular or extra-spinal involvement, including symptoms of the eye, heart, lung and skin
Examples of Genetic factors
HLA-B27
TLR2 variants (ReA)
IL-23R
ERAP1

Modified from Ehrenfeld, 2012, Reveille, 2012, Tsui et al., 2008

Despite the fact that ReA has been identified several decades ago and it has been studied widely, the pathogenic mechanism behind the disease is still unknown. Recently, the understanding of the disease pathogenesis has been improved and it is known that ReA is triggered by a combination of host susceptibility factors and environmental triggers (Alanärä et al., 2012, Hill Gaston and Lillicrap, 2003). The development of the disease is mediated by the host-bacteria interaction. However, efficient treatment of the chronic infection caused by the persisting bacteria is needed and further studies are required to improve both diagnostics and treatment (Reviewed in Rihl et al., 2006).

2.2.1 ReA triggering bacteria

ReA develops often as a complication of certain gastrointestinal, urogenital or respiratory tract infections (Table 3). ReA has sometimes been considered as a sterile arthritis and it is believed that active infection is not present at the time ReA symptoms occur. However, there is increasing evidence showing that bacterial compartments can be detected in the synovial tissue or fluid of ReA patients (Gérard et al., 1998, Gérard et al., 2000, Granfors et al., 1989, Granfors et al., 1990, Granfors et al., 1998). Peripheral blood mononuclear cells (PBMCs) may transport bacterial antigens from gut to joints through endothelial cell layer (Kirveskari et al., 1998). There is also evidence that even viable but non-cultivable bacteria may persist in joints in *Chlamydia*- and *Salmonella*-triggered ReA (Nanagara et al., 1995, Reviewed in Rihl et al., 2006). Persisting bacteria may function as a reservoir of the microbial antigens sufficient to maintain inflammatory response and lead to joint injury (Inman and Chiu, 1998). This is supported by studies showing that patients suffering from *Salmonella*- or *Yersinia*-triggered ReA have high and persisting IgA and IgG antibody response (Granfors et al., 1980, Mäki-Ikola et al., 1991).

All ReA-triggering bacteria share some common features. They are aerobic (*Salmonellae* are facultative anaerobic), Gram-negative, invasive, and either obligate or facultative intracellular bacteria containing LPS at their outer membrane. Typically ReA-triggering infections are caused by *Campylobacter, Chlamydia*, *Salmonella*, *Shigella* or *Yersinia* (listed in Table 3). These bacteria usually cause infection at the gastrointestinal, urogenital or respiratory tract, where they trigger a local inflammation. From the mucosal areas, bacteria are transported elsewhere in the body by monocytes (Kirveskari et al., 1998) that are, together with macrophages, important for the first line host response.

Despite the host defense mechanisms, the bacteria might be capable to evade immunological defense and persist either at the primary site of infection or at the joint. This idea is supported by the fact that bacterial compartments like LPS and even nucleic acids (deoxyribonucleic acid, DNA, and ribonucleic acid, RNA) from the triggering bacteria have been found from synovial fluids (SFs) of ReA-patients (Gaston et al., 1999, Gérard et al., 1998, Gérard et al., 2000, Granfors et al., 1989, Granfors et al., 1990, Granfors et al., 1998). It has been shown in a U937 monocytic macrophage cell line model that *S.* Enteritidis is able to survive and replicate within

HLA-B27 positive monocytic macrophages (Laitio et al., 1997, Penttinen et al., 2004). However, viable *Salmonella* has not been detected from SF of ReA patients but, instead, bacterial components and even bacteria with LPS but without DNA have been found (Granfors et al., 1990, Nikkari et al., 1999). The persistence of viable bacteria cannot be excluded as intracellular *Salmonella* resides in Salmonella containing vacuoles (SCVs). Yu et al. proposed that in HeLa cells, *S.* Typhimurium may reside and replicate in damaged SCVs and acquire nutrients from autophagies (Yu et al., 2014). Also forms of intracellular *C. trachomatis* and *pneumoniae* have been detected in arthritis patient synovial tissue (Gérard et al., 1998, Gérard et al., 2000, Nanagara et al., 1995). ReA triggering bacteria *Chlamydiae*, *Salmonellae* and other enterobacteriaceae e.g. *Shigella* may enter a dormant state in macrophages when exposed to stress (Berthelot et al., 2013). During the dormant state, only 5% of the genome is expressed and the bacteria cannot be cultured. It has been suggested that the dormant state can be promoted by host cell molecule misfolding (Berthelot et al., 2002). However, at the moment, it can be only speculated whether the expression of misfolded HLA-B27 molecules promote the phenomena and thus enable the bacterial persistence linked to SpAs (Berthelot et al., 2013). Moreover, infection with the latent or dormant bacteria might explain the association between chronic inflammatory bowel disease and SpA as it has been hypothesized that infected macrophages or dendritic cells in the submucosa might migrate to the joints and induce chronic TNFα release (Berthelot et al., 2013).

The genus *Salmonella* belongs to the *Enterobacteriaceae* family. *Salmonella* genus consists of two species, *S. enterica* and *S. bongori*. Typically the *Salmonella* serovars causing disease in humans and domestic animals, belong to *S. enterica* subspecies *enterica*. The *S. enterica* serovars are common in animals and environmental sources and cause a wide variety of diseases both in human and animals (Reviewed in Fàbrega and Vila, 2013). *S.* Typhi causes a severe systematic typhoid fever common in the developing world. Non-typhoidal Salmonellosis, typically manifested as gastrointestinal enteritis, can be caused by several *Salmonella* serovars, e.g. *S.* Typhimurium and *S.* Enteritidis. According to Centers for Disease Control and prevention, there are annually 40,000 reported salmonellosis cases in the United States. In 2012, a total of 92,916 salmonellosis cases were reported by the 27 EU member states and there were 40.8 confirmed cases per 100 000 in Finnish population (Eurosurveillance, 2012). It has been suggested that the real number of cases is 30-fold greater or even more as not all are diagnosed and the milder cases are not reported (Reviewed in Fàbrega and Vila, 2013). Futhermore, it has been estimated that there are over 90 million episodes and over 150 000 deaths per year worldwide (Reviewed in Haeusler and Curtis, 2013). *S.* Enteritidis and *S.* Typhimurium are the most common but not the sole ReA-triggering *Salmonella* serovars, as over 20 out of approximately 2500 serovars have been identified as possible ReA pathogens (Petersel and Sigal, 2005). *Salmonella* induced ReA attack rate varies between 1% and 30% in different studies (Reviewed in Carter, 2010, Hannu et al., 2002, Reviewed in Petersel and Sigal, 2005).

Table 3. Reactive arthritis–triggering bacteria and association with HLA-B27

Typical ReA-triggering bacteria	**HLA-B27**	
Gastrointestinal infections		
Salmonella		
S. Enteritidis	Yes	
S. Typhimurium		
Shigella		
S. flexneri	Yes	
S. dysenteriae		
S. sonnei		
Yersinia sp.		
Y. enterocolitica	Yes	
Y. pseudotuberculosis		
Campylobacter		
C. jejuni	Yes	
C. coli		
Urogenital infections		
Chlamydia trachomatis	Yes	
Respiratory infections		
Chlamydia pneumoniae	Yes	
Suggested ReA-triggering bacteria	**HLA-B27**	**Reference**
Gastrointestinal infections		
Clostridium difficile	Yes	1
Escherichia coli	No*	2, 3
Diarrhogenic strains		
Urogenital infections		
Ureaplasma urealyticum	Yes	1, 2
Mycoplasma	No	
M. hominis	No	1
M. genitalium (role in reactive arthritis still unsettled)		2
Respiratory infections		
Group A β-hemolytic Streptococcus (causes acute rheumatic fever, but has been described to cause "reactive-like" arthritis)	No	1, 2
Others	No	1
Borrelia, *Staphylococcus sp.*, *Bartonella henselae*, *Brucella abortus/mellitensis*, *Leptospira*		

Modified from [1.] Rihl 2006, [2.] Hannu 2011, [3.] Schiellerup 2008

2.2.2 Genetic factors associated with ReA

The development of ReA involves both genetic and infectious factors. As bacterial compartments e.g. LPS and nucleic acids are able to persist in ReA patients (Gaston et al., 1999, Granfors et al., 1990, Granfors et al., 1998), continued host response and ineffective bacterial elimination are thought to play an important role in ReA pathogenesis. Thus the host-pathogen interaction is of importance in the disease outcome. Besides HLA-B27 (discussed later in Chapter 2.3), other genetic factors also may modulate host response and play a role in ReA. For example, single nucleotide

polymorphisms (SNPs) in Toll-like receptor 2 (TLR2) are associated with *Salmonella* serovar Enteritidis-triggered ReA (Tsui et al., 2008). However, in the same cohort study, TLR4 genetic variants were not found to be associated with acute ReA although both the innate and adaptive immune responses function through lipopolysaccharide LPS-TLR4 signaling throughout the infection caused by Gram-negative bacteria (Vazquez-Torres et al., 2004). This finding is supported by another study stating that common TLR4 polymorphisms associated with increased risk of severe infections are not associated with neither ReA nor AS in Hungarian population (Gergely et al., 2006). Also HLA-DR1, HLA-B60, HLA-B61 and interleukin 1 (IL-1) gene cluster are associated to AS susceptibility (Brown et al., 1998, Timms et al., 2004, Wei et al., 2004). In addition, there is a genetic association between endoplasmic reticulum (ER) aminopeptidase 1 (*ERAP1*) polymorphisms and AS (Harvey et al., 2009). Also high ERAP1 protein expression has been linked to AS pathogenesis in patient dendritic cells suggesting that ERAP1 might promote AS (Campbell et al., 2011). Additionally, TNF-a6 allele linked to low TNFα secretion is associated with HLA-B27 and ReA and the IL-23 receptor gene (*IL23R*) polymorphisms are found to contribute to AS pathogenesis (Burton et al., 2007, Flores et al., 2003).

2.3 HLA-B27

HLA-B27 is a major histocompatibility complex class I (MHCI) molecule. MHCI molecules are cell surface proteins expressed in all nucleated cells, for example in leukocytes where they were first demonstrated. MHCI molecules are a part of the adaptive immune system, as the main function of the molecules is to present antigenic peptides to cluster of differentiation 8 antigen positive (CD8+) cytotoxic T cells. MHCIs display fragments of cell's protein content and enable the detection of proteins that are not self-derived or are abnormal, such as viral and bacterial peptides. This makes it possible to destroy infected or for example tumor cells while leaving neighboring uninfected or normal cells intact (Reviewed in Campbell et al., 2012). HLA-B27 is a widely studied risk factor for SpAs. The prevalence of HLA-B27 varies between different populations. It is highest in some indigenous populations, for example the HLA-B27 prevalence is 53% in the Pawaia tribe in Papua New Guinea. In Scandinavia, HLA-B27 is also common (15%–25%) but in Japan, the prevalence is only 1 % (Reviewed in Stolwijk et al., 2012). There are several HLA-B27 subtypes and not all are equally associated with SpAs. The most common subtypes (HLA-B*2705, B*2702, B*2704 and B*2707) are clearly associated with SpA whereas B*2706 and B*2709 are not (Reviewed in Reveille, 2006). The relation of HLA-B27 is reported to be up to 70% in patients with ReA (Reviewed in Stolwijk et al., 2012). However, it has been reported that HLA-B27 not only correlates with the risk of developing ReA symptoms but also the development of more severe and prolonged symptoms (Ekman et al., 2000).

HLA-B27 molecule comprises of an HLA HC non-covalently associated with a β2-microglobulin (β2m) light chain and a short peptide (usually 8-10 amino acid residues) derived from self-proteins, viruses or bacteria (Payeli et al., 2012). The heterodimer

complexes of nascent HC and β2m are formed in the endoplamic reticulum (ER; MHCI assembly described in Figure 1.). Peptide binds to it, and formed heterotrimers express through golgi to the cell surface. In early folding stage, during glycosylation, newly synthesized MHCI HCs associate with ER chaperone calnexin (CNX). CNX enhances glycoprotein folding, reduces aggregation, and protects the HC from ER-associated degradation (ERAD) (Vassilakos et al., 1996). ERAD is a cellular quality control process by which misfolded proteins are degraded after retranslocation to the cytosol (Hampton and Sommer, 2012). ER stress protein 57 (ERp57) oxidoreductase mainly functions in the ER. It is recruited by CNX and catalyses MHCI molecule intrachain disulfide bond formation (Lindquist et al., 2001) ensuring the proper folding. MHCI HCs are released from CNX/ERp57 cycle after the right conformation has been achieved. If the folding process is not completed and the released protein remains in unmature conformation, a protein folding sensor glycoprotein glucosyltransferase (UGGT) can direct it to reglucosylation and further rounds of CNX binding and folding may occur (Reviewed in Antoniou et al., 2011a).

After β2m association to HC CNX is replaced by its homologue, another glycoprotein folding chaperone, calreticulin (CRT). Chaperone molecule tapasin associates with transporter associated with antigen processing (TAP) heterodimer (TAP1 and TAP2) and ERp57 is conjugated to tapasin. The complex is associated with CRT/HC/β2m as CRT recruits ERp57 and tapasin bounds to the HC (Reviewed in Peaper and Cresswell, 2008). Thus the peptide loading complex (PLC) is formed. Tapasin is essential for PLC formation and it facilitates the formation of peptide/HC/β2m complexes (Reviewed in Colbert et al., 2010). It has been suggested that tapasin stabilizes the empty MHCI peptide binding groove while CRT protects it from degradation (Reviewed in Colbert et al., 2010, Dong et al., 2009). CRT is also an important quality control molecule since it can transit with suboptimally loaded MHCI molecules to the Golgi apparatus and retrieve these molecules back to the ER (Howe et al., 2009). Tapasin/ERp57 interaction is required to support optimal PLC activity and recruiting MHCI molecules (Dong et al., 2009). TAP heterodimer recruits tapasin and transports peptides to the ER from the cytosol.

Peptides loaded into MHCI molecules are derived from the proteins degraded at the proteasome within the cytosol and transported to the ER with TAP. Besides optimal length 8-13, the peptides must have the correc t binding properties in order to be loaded onto MHCI peptide binding groove with the assistance of the PLC. ERAP1 cuts peptides to a suitable length for HLA-B27 binding (Reviewed in McHugh and Bowness, 2012). PLC ensures that only optimal peptides are loaded and it is essential for presenting MHCI molecules at the cell surface. When the PLC cannot form, or it is formed inadequately, HC/β2m/peptide complexes may still be expressed on the cell surface. However, the amount of molecules is reduced (Reviewed in Peaper and Cresswell, 2008) and they are often found at the cell surface only transiently, as they may contain suboptimal peptides that readily dissociate (Reviewed in Colbert et al., 2010).

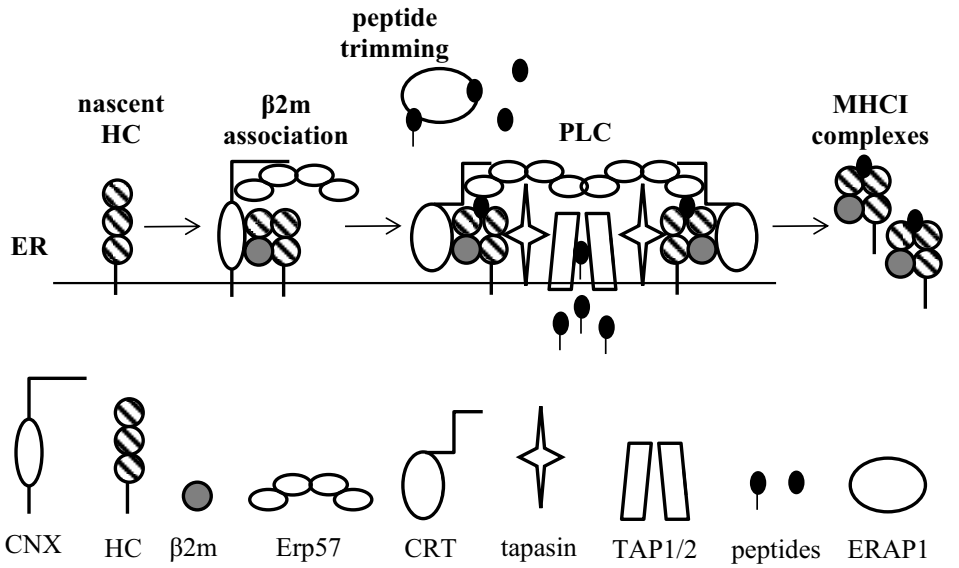

Figure 1. Major histocompatibility complex **I (MHCI) Assembly in the endoplasmic reticulum (ER).** After β2-microglobulin (β2m) association to the heavy chain (HC), formed heterodimer is loaded with the peptide through peptide loading complex (PLC). Modified from Peaper and Cresswell, 2008 and Blum et al., 2013.

2.3.1 Role of HLA-B27 in the pathogenesis of ReA and other SpA

MHCI molecules are a highly unusual group of proteins as they may attain several different conformations during their assembly. Moreover, HLA-B27 has unique features among MHCI molecules. These include peptide binding specificity, a tendency to misfold, and a tendency to form HC homodimers at the cell surface (Reviewed in Colbert et al., 2014). HC dimerization may take place in two distinct cellular locations. Dimerization of the newly synthesized HCs occurs in the ER and later β2m-free HCs may dimerize at the cell surface. Because these events have very different implications Colbert et al. have suggested that the term misfolding refers to all aberrant events occurring in the ER and events at the cell surface should be referred as dimerization (Reviewed in Colbert et al., 2010). This classification is followed hereafter. ER chaperones assist in the conformational assembly and regulate the degradation of misfolded proteins. The correct folding and the final structure of the complex are determined by the peptide bound to the peptide binding groove (Reviewed in Antoniou et al., 2011a, de Castro, 2009). The chaperone machinery should be able to distinguish the different but normal conformations that MHCI molecules can attain during the folding process from the structures that indicate that molecules are terminally misfolded (Reviewed in Antoniou et al., 2011a).

The unique features of HLA-B27 have inspired different lines of research. At the moment, there is a consensus that the three main theories might explain the role of HLA-B27 in the pathogenesis of SpA. Firstly, peptide presentation to CD8+ T cells

(arthritogenic peptide and molecular mimicry theories). Secondly, HLA-B27 HC misfolding and thirdly, recognition of the abnormal forms (e.g. aberrant dimers) of the HLA-B27 HCs by immune effector cells (Reviewed in Colbert et al., 2014). Most likely all these aspects confer to the interplay between SpA susceptibility and HLA-B27.

2.3.1.1 Arthritogenic peptides

Pathogenic peptides derived from the pathogenic bacteria or specific arthritogenic peptides found in joints are presented to self-reactive T cells. In addition to arthritogenic peptides originating from chlamydia- and yersinia outer membrane proteins (OMPs), also immunogenic HLA-B*2705-binding peptides from salmonella OMP in patients with ReA/uSpA have been identified (Singh et al., 2013). Alternatively peptides stimulate T cells that are able to recognize a self-peptide associated to HLA-B27. It is recently shown that self-peptides containing Gln at the P2 anchor motif that show significant homology to arthritogenic bacterial sequences (differ from each other by 2–3 residues) and bind specifically to HLA-B27 subtypes B*2704 and B*2705 that are associated with SpA (Infantes et al., 2013).

2.3.1.2 Misfolding of the HLA-B27 heavy chains

HLA-B27 folding is unusually slow in comparison with most MHCI molecules (Mear et al., 1999). Misfolding events occur in the ER (Reviewed in Colbert et al., 2010). Unlike misfolding of the other HLA alleles, HLA-B27 misfolding occurs at the presence of an intact antigen processing and assembly pathway and can also be detected at the presence of β2m and a normal amount of peptide cargo (Mear et al., 1999, Dangoria et al., 2002). HLA-B27 misfolding is associated with unusual peptide binding groove, B pocket, structure (Reviewed in Colbert et al., 2014). Ineffective peptide loading plays a role for HLA-B27 since significantly increased peptide concentration is needed to achieve proper binding. Furthermore, peptide binding is needed to form proper final conformation (Reviewed in Colbert et al., 2014). Usually misfolded HCs are exported to the cytosol to be processed by ERAD (Reviewed in Colbert, 2004). However, they may escape the quality control process and can accumulate in the ER and trigger a stress response, or they may traffic to other parts of the cell (Mear et al., 1999).

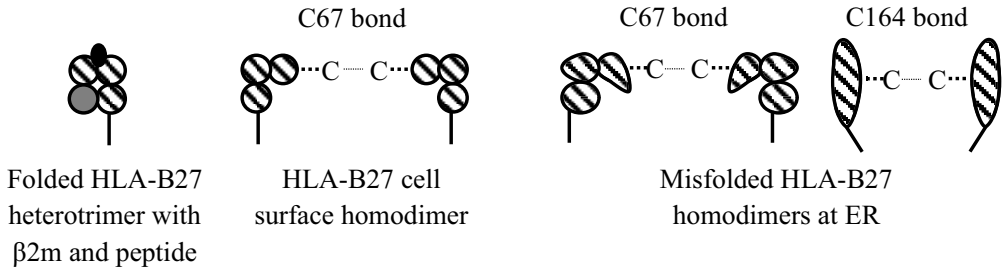

Figure 2. Schematic picture of different HLA-B27 forms.
Modified from Antoniou et al., 2011b

HLA-B27 peptide binding groove has conserved amino acid composition (His9, Thr24, Glu45, Cys67, Lys70, Ala71, and Gln97) compared with other HLA-B alleles. Glutamic acid at position 45 (E45), and cysteine at position 67 (C67) have been shown to be important in HLA-B27 functionality (peptide binding, cell surface expression, and CTL recognition) (Buxton et al., 1992, Mear et al., 1999, Dangoria et al., 2002). It has been suggested that HLA-B27 misfolding occurs as a consequence of slow HC folding and prolonged exposure of unpaired cysteine residues 67 and 164 at the peptide binding groove to the oxidizing environment of the ER (Antoniou et al., 2004, Dangoria et al., 2002). Cys67 is more significant in dimerization at the cell surface and Cys164 in misfolding (Reviewed in Antoniou et al., 2011b). Aberrant inter- and intra-chain disulfide bonds are formed and HC dimers presenting varying degrees of folding are composed (Lenart et al., 2012). These HCs complexes have prolonged association with the ER chaperone called binding immunoglobulin protein (BiP) (Dangoria et al., 2002, Tran et al., 2004). BiP is known to form a stable association with proteins that misfold and the retranslocation of misfolded proteins from ER to cytosol involves BiP. Association with BiP may prevent the aggregation of the unfolded protein and lead to ERAD (Reviewed in Guerriero and Brodsky, 2012). However, HLA-B27 is upregulated upon infection. It has been shown that ERAD is not sufficient to clear misfolded HCs when they accumulate in the ER (Turner et al., 2007). This might result ER stress and the activation of ER overload response (EOR) and unfolded protein response (UPR). Overexpression of misfolding HLA-B27 with human β2m induces an inflammatory disease closely resembling SpA in rats (Hammer et al., 1990). In the transgenic rat model, an ongoing UPR is detected after HLA-B27 upregulation (Turner et al., 2005, Turner et al., 2007). However, whether UPR is activated in human cells expressing HLA-B27 is not clear. Nevertheless, it has been shown that UPR occurs during monocyte to macrophage differentiation (Dickhout et al., 2011). This protects the cell from later ER stress signals that trigger UPR and macrophage cell death as UPR during differentiation increases the amount of molecular chaperones in the ER. UPR also leads to altered cytokine production regulating both pro- and anti-inflammatory responses. In contrast to well characterized UPR, the mechanism of EOR is still poorly defined (Reviewed in Antoniou et al., 2011b). It has been suggested that EOR at least partially overlaps with the UPR response (Reviewed in Colbert, 2004). EOR is linked to the activation of the NF-κB and thus it mediates the proinflammatory cytokine response (Pahl and Baeuerle, 1995). Taken together, it has been suggested that three characteristics of HLA-B27 support the conclusion that it misfolds (Reviewed in Colbert et al., 2010). First, HLA-B27 HCs accumulate in the ER and undergo ERAD; second, they self-associate into complexes in the ER; and third, they exhibit a prolonged association with BiP. Misfolding is proved to be important in the pathogenesis of SpA, as in addition to transgenic rat models where misfolding is linked to SpA, it was recently shown that HLA-B27 misfolding occurs in the gut of HLA-B27 positive AS patients (Ciccia et al., 2013).

2.3.1.3 HLA-B27 dimerization

HLA-B27 HC can form β2m-free HC homodimers (Allen et al., 1999b) found at the cell surface. Besides dimers, free HLA-B27 HCs (monomers) can also be formed and

found at the cell surface (Reviewed in McHugh and Bowness, 2012). It is not clear how they are formed but rather than expressing ER-originated HLA-B27 homodimers to the cell surface, aberrant forms are most likely formed from unstable HLA-B27 heterotrimeric complexes already found at the cell surface (Bird et al., 2003). It has been suggested that HLA-B27 peptide loading is less tapasin-dependent than other MHCI molecules as HLA-B*2705 molecules presenting viral peptides are found on the cell surface in the absence of tapasin (Peh et al., 1998). However, peptides are more weakly bound and may dissociate at the cell surface. Peptide dissociation leads to detaching of the β2m leaving the HC free (Reviewed in McHugh and Bowness, 2012). Aberrant homodimer formation may require involvement of an endosomal recycling pathway as the partial unfolding of the molecules is possible in the acidic environment of the endosome (Bird et al., 2003). Homodimerization can be triggered by for example bacterial infection or stress signal. Peptide and/or β2m dissociate from the complex, the unpaired cysteine residue at position 67 (Cys67) at the HLA-B27 HC becomes exposed and a disulphide bond is formed between the two cysteines (Allen et al., 1999b, Campbell et al., 2012, McHugh and Bowness, 2012). This forming of the disulphide bond through the highly reactive cysteine is unusual, although not unique feature of the HLA-B27 and critical for homodimer formation (Reviewed in McHugh and Bowness, 2012). Homodimers are capable to maintain at least partially functional conformation and thus bind and present peptides (Allen et al., 1999b, Reviewed in Allen et al., 1999a). In addition to HLA-B27 recognition by T-cell receptors (TCRs), HLA-B27 molecules and dimers are recognized by innate immunoregulatory receptors on natural killer and T-cells, including killer cell immunoglobulin-like receptors (KIRs) and leukocyte immunoglobulin-like receptors (LILRs), a phenomenon that may lead to an aberrant pro-inflammatory response (Reviewed in Campbell et al., 2012 and McHugh and Bowness, 2012). Moreover, in comparison to classical HLA-B27 heterotrimers, homodimers and monomers interact in part with the different subtypes of these receptors leading to an aberrant activation of the cells by stimulating the production of pro-inflammatory mediators (Reviewed in Ambarus et al., 2012, Kollnberger and Bowness, 2009). For example, HC dimers and multimers are stronger ligands for LILRB2 (formerly ILT4) than heterotrimers (Giles et al., 2012) but do not bind to LILRB1 (ILT2) (Kollnberger et al., 2002). It has been suggested that this aberrant interaction could play a role in the pathogenesis of SpAs (Giles et al., 2012). HLA-B27 dimer expressing cells stimulate the survival, proliferation and production of IL-17 of the KIR3DL2+ expressing CD4+ T cells (Bowness et al., 2011). Also, this subset of T cells is enriched in the peripheral blood and SF of SpA patients constituting a possible therapeutic target in SpA.

2.4 Host-microbe interaction

All organisms try to defense themselves against external threats like pathogens, whereas pathogens try to the exploit the host cell by using it to enable proliferation. Thus the interaction between pathogen and host cell is a complex train of events. Usually, pathogens are recognized and internalized by antigen-presenting cells (APCs) e.g. macrophages and dendritic cells. There is evidence that the expression of HLA-

B27 influences on the host-pathogen interaction. It has been shown that the expression of HLA-B27 modulates the intracellular survival of *Salmonella* in murine L cells and human monocytic cell line U937 cells (Laitio et al., 1997, Virtala et al., 1997). Impaired bacterial elimination is associated with the glutamic acid at position 45 in the HLA-B27 peptide binding groove linked to the misfolding feature of HLA-B27 HCs (Penttinen et al., 2004). Host cell HLA-B27 expression correlates with enhanced and prolonged NF-κB activation and TNFα secretion in U937 cells after stimulation with *Salmonella* LPS (Penttinen et al., 2002). Moreover, it has been shown that the expression of HLA-B27 HCs modulates *Salmonella* gene expression possibly leading to increased survival and replication (Ge et al., 2012).

Crucial steps for bacteria survival are adhering to the host cell, replication within the cell and dissemination. In order to survive and replicate, *Salmonella* enters to the host cells, preferentially non-permissive immune cells like macrophages (Reviewed in Garai et al., 2012, García-del Portillo et al., 2008). *Salmonella* may internalize to the host cell by various methods; epithelial cells engulf *Salmonella* by macropinocytosis whereas macrophages phagocytose *Salmonella* (Reviewed in Garai et al., 2012). *Salmonella* invasion to the host cell requires disruption and rearrangement of cellular actin (Reviewed in Brodsky and Medzhitov, 2009). Bacterial virulence factors, also called effectors, are delivered to the host cell cytosol by a specialized secretion system. In *Salmonella enterica* this system is called type III secretion system (T3SS). *Salmonella* effectors activate G-proteins that induce the generation of actin-rich membrane structures (Reviewed in Bhavsar et al., 2007). Within macrophages, *Salmonella* resides in SCVs where it is possible to hide from the immune detection. *S. enterica* T3SS effector protein SigD (also called SopB) catalyses the dephosphorylation of phosphoinositides required for the efficient formation of SCVs (Terebiznik et al., 2002) whereas SopA may promote *Salmonella* escape from the SCVs (Angot et al., 2007). However, SCVs form in three stages (Reviewed in Steele-Mortimer, 2008). At the early stage, up to 30 minutes post infection, *Salmonella* resides within simple vacuole or phagosome. The intermediate stage, from 30 minutes to approximately 5 hours post infection, is characterized by Lamp enriched tubules. After that, at the late stage, bacterial replication is initiated and *Salmonella*-induced filaments are formed around the SCV throughout the cytosol (Reviewed in Steele-Mortimer, 2008). SCVs interrupt the host endosomal pathway, get juxtanuclear and exploit the Golgi apparatus in order to get nutrition and enable bacterial replication (Guignot and Servin, 2008). Bacterial virulence factors, encoded by the Salmonella pathogenity islands (SPI), work against host immune responses trying to alter the response in favor of the pathogen. SPI1 and SPI2 are regarded as the two major virulence determinants of *Salmonella* (Pezoa et al., 2013) although SPI3-5 are also involved. SPI1 mediates bacterial invasion by mediating actin cytoskeletal rearrangements and SPI2 mainly intracellular survival and replication (Reviewed in Fàbrega and Vila, 2013). Moreover, for example SPI19 was recently found to be essential for intracellular *Salmonella* survival in macrophages (Blondel et al., 2013). *S. enterica* SPI2 virulence factors induce a reduction of antigen presentation on MHCII molecules in dendritic cells (Cheminay et al., 2005) and *S.* Typhimurium SPI1 and

SPI2 factors downregulate MHCII expression on porcine macrophages (Van Parys et al., 2012).

Bacteria attack the intracellular signaling pathways of the host, for example NF-κB and mitogen activated protein kinase (MAPK) pathways (Reviewed in Brodsky and Medzhitov, 2009). Many intracellular pathogens interfere phosphorylation by kinases (e.g. MAPKs) or dephosphorylation by phosphatases (Reviewed in Bhavsar et al., 2007). *Yersinia* effector YpkA has structural and functional similarities to eukaryotic serine/threonine kinases and in the host cell, it is activated by autophosphorylation (Prehna et al., 2006). YpkA modulates the cytoskeleton (Prehna et al., 2006). *Shigella flexneri* effector OspG and *Yersinia* spp. effectors YopP and YopJ prevent NF-κB activation by preserving IκB from degradation (Reviewed in Angot et al., 2007 and Bhavsar et al., 2007). *S. flexneri* OspF dephosphorylates extracellular signal–regulated kinase (ERK2) preventing its activation and downstream signal transduction inhibiting the NF-κB-dependent transcription (Arbibe et al., 2007). *Yersinia* spp. YopP/J inhibit p38 and c-Jun NH2-terminal kinase (JNK) MAPK phosphorylation leading to reduced antigen uptake and altered antigen presentation by dendritic cells (Autenrieth et al., 2007). YopP/J also induce apoptosis, possibly via reduced NF-κB signaling whereas *Shigella* spp. IpaB and *S. enterica* SipB and TlpA induce apoptosis through caspase 1 activation (Reviewed in Bhavsar et al., 2007 and Scott and Saleh, 2007). *Salmonella* TlpA (Toll-interleuin 1 receptor [TIR]-like protein A) also inhibits the activity of mammalian TIR-containing proteins TLR4, IL-1 receptor, and myeloid differentiation factor-88 (MyD88) and the downstream target NF-κB (Newman et al., 2006). TlpA is found to be crucial for intracellular survival in human macrophages and it is required for full virulence (Newman et al., 2006). Moreover, *Shigella* and *Salmonella* TTSS effectors are able to irreversibly inactivate their host MAPK pathways by removing the phosphate from the phosphothreonine in the activation loop (Zhu et al., 2007).

2.5 Infection and ER Stress

Protein folding, assembly and quality control takes place in ER. Unfolded and misfolded proteins may accumulate in the ER if the protein folding machinery is overloaded or disturbed. This causes the activation of UPR that increases the protein folding capacity by inducing the synthetization of proteins involved in the folding machinery and reduces the loading of the newly synthetized proteins into the ER by inducing a translational arrest (Reviewed in Walter and Ron, 2011). Also ERAD and protein degradation by lysosome-mediated autophagy are activated during ER stress (Ogata et al., 2006).

There are three molecules acting as primary UPR signal transducers (Figure 2.). Activating transcription factor 6 (ATF6), PERK (double-stranded [ds] RNA-activated protein kinase [PKR]-like ER kinase) and IRE1 (inositol requiring enzyme 1), activate three separated but collaterally acting UPR branches (Reviewed in Walter and Ron, 2011). ATF6, PERK and IRE1 are ER-resident transmembrane proteins that are bound to BiP in order to maintain inactive state (Reviewed in Colbert et al., 2010). When

unfolded proteins accumulate into the ER, ATF6 is translocated to the golgi and activated by cleavage (Reviewed in Hollien, 2013). The remained cytosolic fragment moves to the nucleus and activates the transcription of UPR target genes including BiP and CCAAT/enhancer-binding protein (C/EBP)-homologous protein (CHOP) (Ogata et al., 2006). In ER stress, PERK oligomerizes and is autophosphorylated (Reviewed in Tabas and Ron, 2011). PERK phosphorylates eukaryotic translation initiation factor 2α (eIF2α) causing the inactivation of it and arrest in the messenger RNA (mRNA) translation. However, some proteins encoded by specific mRNAs containing short open reading frames in their 5′ untranslated regions (5'UTRs) can be upregulated when eIF2α is limited (Reviewed in Hollien, 2013). One such protein is transcription factor ATF4 that regulates genes involved in ER functions. Expression of CHOP that controls genes involved in apoptotic functions, and growth arrest and DNA damage-inducible protein 34 (GADD34) negatively regulating eIF2α are regulated by ATF4 (Reviewed in Hollien, 2013, Walter and Ron, 2011 and Wang and Kaufman, 2012). IRE1 is widely expressed from yeast to humans. It has two homologues, IREα and IREβ. IREα is expressed in all cell types and mediates UPR. IRE1 oligomerization leads to conformational changes causing the activation of ribonuclease function. Activated IRE1 initiates splicing of X-box binding protein (XBP1) intron and the protein product activates the transcription of genes encoding factors involved in ER functions like protein folding and degradation (Reviewed in Guerriero and Brodsky, 2012). It has been proposed that IRE1 might also directly bind to the peptide binding pocket of misfolded proteins after BiP dissociation and activate UPR (Reviewed in Guerriero and Brodsky, 2012). Moreover, XBP1 abnormalities are linked to intestinal inflammation in a mouse model (Kaser et al., 2008).

UPR can trigger apoptosis in case ER stress is not solved properly. The exact mechanism is still unknown but multiple factors are involved. IRE1α triggers TNFα-dependent apoptosis via TNF receptor-associated factor 2 (TRAF2), apoptosis signal-regulating kinase 1 (ASK1) and JNK (Reviewed in Colbert et al., 2010). PERK and ATF6 induce apoptosis via CHOP induction (Reviewed in Tabas and Ron, 2011 and Colbert et al., 2010). CHOP has a dual role in transcription, it upregulates the transcription of proapoptotic genes and downregulates antiapoptotic genes (Chiribau et al., 2010). For example, CHOP dimerizes with C/EBPβ isoform liver-enriched inhibitory protein (LIP) in order to suppress a pro-survival protein *Bcl-2* in mouse fibroblasts (Chiribau et al., 2010). CHOP also induces oxidative stress by causing the accumulation of reactive oxygen species (ROS) that in turn activate PKR that amplifies CHOP expression (Reviewed in Tabas and Ron, 2011). Moreover, PKR is activated during sustained ER stress and it induces the eIF2α regulated attenuation of protein synthesis (Lee et al., 2007). Interestingly, in macrophages exposed to LPS and experiencing prolonged ER stress, CHOP-induced apoptosis may be avoided by selective suppression TLR signaling (Reviewed in Tabas and Ron, 2011, Woo et al., 2009).

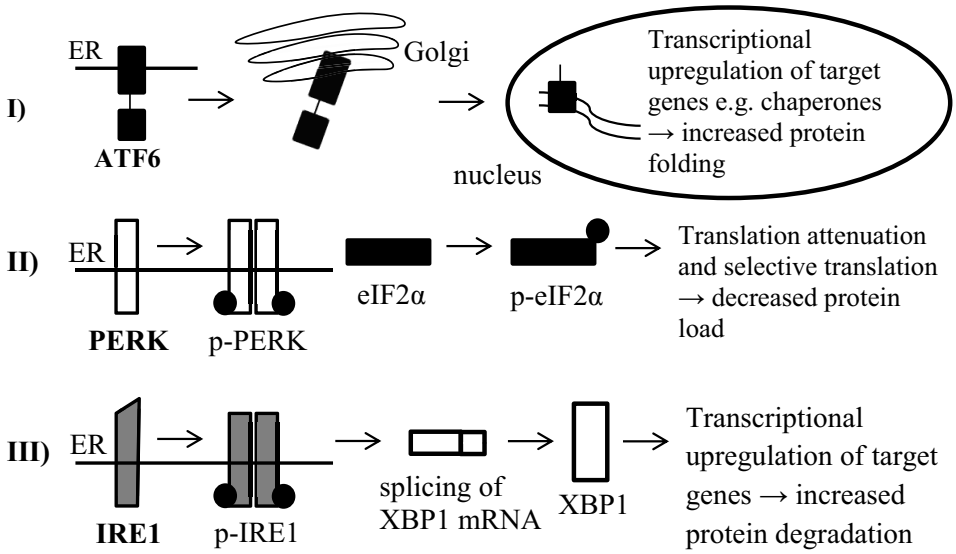

Figure 3. An overview of unfolded protein response (UPR) signaling cascades activated by endoplasmic reticulum (ER) stress. Activating transcription factor 6 (ATF6), PKR-like ER kinase (PERK) and inositol requiring enzyme 1 (IRE1) are primary UPR signal transducers that activate three separate, but collaterally acting UPR branches.

2.6 Cytokines

The pro-inflammatory cytokine production is induced during the early immune response and is followed by the production of anti-inflammatory cytokines. Cytokines play an important role in the elimination of the intracellular bacteria and moreover, the expression of cytokines like interferon gamma (IFNγ), IL-12 and TNFα is a prerequisite for bacterial elimination. Imbalance between cytokines may inhibit bacterial elimination. Monocytes and macrophages are important in immune response and the number of synovial macrophages in the inflamed joints correlates with the activity of SpA (Baeten et al., 2005). Thus macrophage derived cytokines TNFα, IL-1β, IL-6, IL-10 and IL-23 are of interest in the pathogenesis of SpA (Reviewed in Ambarus et al., 2012). Moreover, it has been suggested that the expression of HLA-B27 modulates cytokine production in macrophages possibly leading to impaired capacity to eliminate intracellular bacteria (Ekman et al., 2002).

Inflammation of peripheral joints is observed both in SpAs and RA. However, pro-inflammatory cytokine levels (TNFα, IL-1β and IL-12p40) detected from patient SF are lower in SpAs than RA (Reviewed in Vandooren et al., 2009). In ReA, low TNFα secretion and HLA-B27 correlate with longer duration and increased disease severity, and possibly contribute to the bacterial persistence (Braun et al., 1999). Measured from ReA patient SF mononuclear cells, low levels of IFNγ and TNFα but high IL-10 secretion is detected (Yin et al., 1997). Interestingly, IL-10, an anti-inflammatory cytokine responsible of immunological tolerance (Reviewed in Ambarus et al., 2012), is found to be responsible for the suppression of IFNγ and TNFα (Yin et al., 1997). Despite the studies

showing that low TNFα levels are related to SpAs, TNFα blocking is highly effective in the treatment of SpAs, especially AS. Although there is some evidence suggesting that TNFα blockade might not prevent new bone formation, it leads to a significant decrease in the disease activity (Reviewed in Hreggvidsdottir et al., 2014 and Sieper, 2011).

There is also evidence that the secretion of TNFα is increased in SpAs as TNFα and IL-1 production was increased in HLA-B27 positive monocytes derived from patients who have had Yersinia-triggered arthritis (Repo et al., 1988). It has been suggested that both high and low TNFα production is a feature of SpA. The contrasting results might also be explained by different methods; whether the PBMCs or for example only monocytes were used. Nevertheless, to date, detailed understanding delineating which cells and forms of TNFα (soluble versus trans-membrane) contribute to the pathologic role of TNFα in human SpA remains to be achieved (Reviewed in Hreggvidsdottir et al., 2014). However, it is known that both *Salmonella* and *Salmonella*-derived LPS induce different cytokine profiles in HLA-B27 and HLA-A2 expressing cells and thus it can be suggested that the expression of HLA-B27 is of importance in cytokine production (Ekman et al., 2002).

In rat macrophages, HLA-B27 misfolding and UPR activation lead to the induction of IL-23 thus linking the protein misfolding, ER stress, and UPR activation with inflammatory disease (DeLay et al., 2009). However, enhanced LPS-triggered IL-23 production but no UPR was detected in AS patient macrophages (Zeng et al., 2011) and recent findings suggest that autophagy, rather than UPR, may be associated with the intestinal modulation of IL-23 production in AS (Ciccia et al., 2013). Moreover, there are several studies suggesting that IL-23 might not contribute to SpA activity in patients as no increased production was detected. Nevertheless, the data suggests that IL-23 production is locally upregulated in human SpAs (Reviewed in Hreggvidsdottir et al., 2014). UPR activation has also been linked to IFNβ and lL-1α production and TNF-α and IL-6 mRNA stability and protein expression in mouse macrophages (Chen et al., 2009, Layh-Schmitt et al., 2013). Increased amount of IL-6 has been detected from SF of arthritis patients and it has been suggested that IL-6 has a protective role in the metabolism of cartilage (Silacci et al., 1998).

2.7 Intracellular signaling

Cell signaling, also referred as signal transduction, is a communication system coordinating cellular activities. By way of both intra- and extracellular signaling, the cell is able to recognize and respond to its environment. Actions needed to accomplish the cell fate are initiated and maintained as message moves on the signaling cascade. Multiple signaling pathways form complex networks that involve various types of molecules; receptors, kinases, phosphatases, cytokines, hormones and many others.

2.7.1 Kinases

Kinases are key regulators of cellular functions in eukaryotic cells. Kinases are proteins catalyzing phosphorylation of other proteins; kinases mediate cellular signaling by regulating substrate proteins by phosphorylation. Kinases are also highly

regulated, usually by phosphorylation. Phosphorylation promotes a conformational change to an active form of a protein. Conformational reorganization eliminates steric hindrance and stabilizes the activation loop allowing the substrate binding (Reviewed in Cuadrado and Nebreda, 2010). Most kinases are serine/threonine (Ser/Thr) kinases, that is to say they phosphorylate Ser or Thr residues on substrates. Some kinases phosphorylate tyrosine (Tyr) residues. Moreover, various kinases phosphorylate all three kinds of amino acid residues and some even phosphorylate other amino acids, like histidine. Reversible phosphorylation regulates enzymatic activity, protein stability, cellular localization, and protein-protein interactions. These actions mediate many cellular processes e.g. transcription, metabolism, apoptosis, inflammation, cell cycle regulation, and cell differentiation.

Most eukaryotic protein kinases (EPKs) share a highly conserved catalytic core. Two motifs, a dynamic activation segment and a GHI (G-helix through the I-helix) helical subdomain are typical EPK structures that separate them from more primitive eukaryotic-like kinases (ELKs) (Reviewed in Taylor and Kornev, 2011 and Taylor et al., 2012). Kinase phosphorylation takes place in the activation segment, either by autophosphorylation or by another kinase, whereas helical subdomain involves docking of protein substrates (Reviewed in Taylor and Kornev, 2011). Kinase activity can also be regulated by activator or inhibitor proteins. Deregulation of kinases has been linked to many human diseases, including developmental and metabolic disorders and cancer (Reviewed in Lahiry et al., 2010 and Manning, 2009). Thus kinases are of interest in research and also drug development as they represent 20% of current drug targets (Varjosalo et al., 2013).

Table 4. Conventional mitogen-activated protein kinases

Conventional MAPKs	SYNONYMS	ISOFORMS	SYNONYMS
p38	RK, CSBP	p38α	MAPK14*
		p38β	MAPK11*, SAPK2
		p38γ	MAPK12*, SAPK3, ERK6
		p38δ	MAPK13*, SAPK4
JNK	MAPK8	JNK1	MAPK8*, SAPK1
		JNK2	MAPK9*, SAPK
		JNK3	MAPK10*, p54bSAPK
ERK1	MAPK3*	ERK1b	
		ERK1c	
ERK2	MAPK1*, MAPK2	ERK2b	
ERK5	MAPK7*, BMK1	ERK5a	
		ERK5b	
		ERK5c	

*Recommended symbol by Human Genome Organisation (HUGO) Gene Nomenclature Committee (HGNC)

2.7.1.1 Mitogen-activated protein kinase pathways

MAPKs are ubiquitous in eukaryotic cells and are involved in many processes, for MAPK activation modulates gene transcription and protein synthesis (Reviewed in Kyriakis and Avruch, 2012). MAPK cascades function parallel and are especially important in the control of cell fate decisions e.g. cell cycle control and programmed cell death (Johnson and Lapadat, 2002, Varjosalo et al., 2013). MAPKs also play an important role in stress reactions and MAPK signaling can be activated by extracellular stress and inflammatory stimuli. MAPK can also be activated by mitogens, growth factors, cytokines, antigens, cell-cell interactions, hormones, PAMPs and danger-associated molecular patterns (DAMPs) (Reviewed in Johnson et al., 2005 and Kyriakis and Avruch, 2012). MAPKs contain common kinase domain (KD) and MAPKs have a specific substrate binding motif also called docking site (Reviewed in Cargnello and Roux, 2011 and Cuenda and Rousseau, 2007).

There are four conventional, three-tiered MAPK pathways; ERK1/2, JNK, p38 kinase and ERK5 pathways (Table 4.). These pathways have three core kinases; MAP kinase kinase kinase (MAP3K), MAP kinase kinase (MAP2K) and MAPK, and the signal is transducted by sequential phosphorylation (Figure 4.) (Reviewed in Kyriakis and Avruch, 2012 and Plotnikov et al., 2011). Moreover, often MAP kinase kinase kinase kinase (MAP4K) acts as an upstream modulator of MAP3K and MAPK activated protein kinase (MAPKAPK) as a downstream activator activated by MAPK. MAPK and MAPKAPK phosphorylate substrate proteins (Reviewed in Plotnikov et al., 2011).

MAP3Ks and MAP2Ks are Ser/Thr kinases. MAP3Ks are activated by phosphorylation or interaction with upstream modulators, small GTP-binding MAP4Ks. There are at least 20 MAP3Ks whereas only 7 MAP2Ks are known. The activity of MAP2Ks is regulated by a variety of MAP3Ks and they are specific towards the MAPK substrates. There are total of 14 conventional and atypical MAPKs (Figure 3.). Conventional MAPKs are activated by simultaneous Tyr and Thr phosphorylation through conserved Thr-X-Tyr motif in the activation loop (Reviewed in Kyriakis and Avruch, 2012). Conventional MAPKs (Table 4.) are present either in the cytoplasm (ERK1/2, ERK5) or both cytoplasm and nucleus (p38 isoforms). In response to activation, MAPKs are shown to partially relocate to the nucleus (Reviewed in Cargnello and Roux, 2011). MAPKs have both cytoplasmic and nuclear substrates. MAPKs are proline (Pro) directed Ser/Thr kinases; MAPKs phosphorylate substrate Ser/Thr residues only if they are followed by Pro residue (Reviewed in Kyriakis and Avruch, 2012). Besides Pro selectiveness, MAPK substrates have a specific docking site that permits a strong and highly selective interaction. There are also atypical MAPKs ERK3/4, ERK7 and NLK. However, these atypical pathways are not three-tiered and ERK3/4 and NLK do not have Thr-X-Tyr activation motif. ERK7 is activated by autophosphorylation through Thr-X-Tyr motif, not by MAP2K (Coulombe and Meloche, 2007).

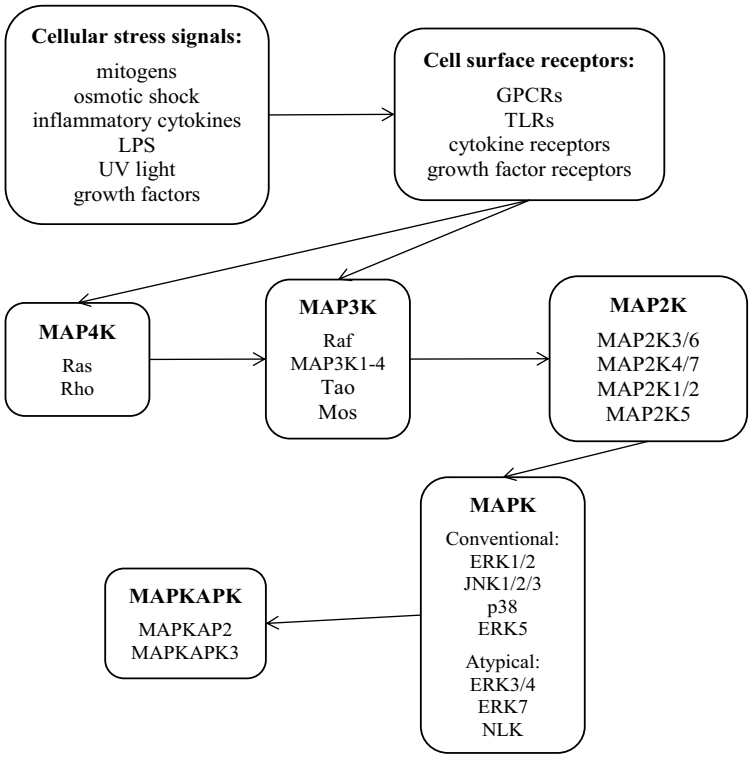

Figure 4. Mitogen activated protein kinase (MAPK) signaling cascade. Examples of molecules involving each step are included.

2.7.1.2 p38 mitogen-activated protein kinase

The p38 pathway is typically activated by stress-related and proinflammatory stimuli like environmental stress, cytokines and PAMPs. Thus p38 MAPKs are important regulators of the immune response; the p38 signaling cascade can be activated by inflammatory cytokines, and in turn, it regulates cytokine expression. All MAPK pathways activate transcription, but p38 pathway also involves in post-transcriptional regulation (Reviewed in Dean et al., 2004). p38 substrates include both kinases and transcription factors including MAPKAPKs like MAPKAPK2-5, mitogen- and stress-activated protein kinases (MSKs) and transcription factors like NF-κB and ATF1,2 and 6 (Reviewed in Nakagawa and Maeda, 2012, Plotnikov et al., 2011). p38 also targets C/EBPβ and human RNA binding protein HuR.

There are four p38 isoforms; α, β, γ and δ (also called MAPK14, MAPK11-13, respectively, listed in Table 4.) that share 60% amino acid sequence identity. p38α and β are ubiquitously expressed in all tissues and cells (Schindler et al., 2007). In contrast, p38γ is mainly expressed in skeletal muscle and p38δ in the testes, pancreas and small intestine (Schindler et al., 2007). p38 isoforms are activated by certain MAP2Ks;

MAP2K6 (MEK6, MKK6) activates all four isoforms, MAP2K3 (MEK3, MKK3) p38α, γ and δ and MAP2K4 (MEK4, MKK4) only p38α and β in some circumstances (Reviewed in Cargnello and Roux, 2011, Qi and Elion, 2005 and Whitmarsh and Davis, 2007). MAP2K3/6 are p38 specific but MAPK2K4 activates also JNK (Reviewed in Cuadrado and Nebreda, 2010). MAP2Ks activate p38s through Thr-Gly-Tyr domain. Phosphorylation of Thr180 is needed for catalysis whereas phosphorylation of Tyr182 only increases the activity by 10-20 % and is more important in autoactivation and substrate recognition. p38 molecule phosphorylated only on Tyr182 is inactive (Askari et al., 2009). p38 autophosphorylation may be stimulated by interaction with adaptor proteins like transforming growth factor-β-activated protein kinase 1 (TAK1)–binding protein 1 (TAB1, also called MAP3K7) or lipidic phosphatidyl inositol analogues (PIAs) and Tyr323 phosphorylation by tyrosine kinase ZAP70 (Ge et al., 2002, Gills et al., 2007, Salvador et al., 2005). For example it is shown that TAB1 directly interacts with p38 and promotes p38 autophosphorylation (De Nicola et al., 2013).

2.7.1.3 p38 pathway function and immune response

Intracellular immune response can be initiated by extracellular, for example microbial, stimuli. PAMPS or DAMPs like heat shock proteins or nucleic acids, engage pattern recognition receptors (PRRs), e.g. TLRs 1-11, at the cell surface. For example, bacterial LPS binds to TLR4 and activates the p38 MAPK signaling cascade. Phosphorylated p38 activates transcription factors like ATF via MAPKAPK2 leading to the production of pro-inflammatory cytokines (Kotlyarov et al., 1999, Winzen et al., 1999). Interestingly, it is noted that while in LPS tolerant PBMC TLR signaling cascade-related gene expression is generally inhibited, p38-related gene expression remains unchanged suggesting involvement of TLR-independent mechanism (Mendes et al., 2011). Moreover, as kinases like p38 are important mediators of the immune response, pathogens produce proteins attacking host cell kinases. Thus pathogens can modulate intracellular immune response through production of virulence factors. *Ehrlichia chaffeensis*, an obligatory intracellular Gram-negative bacterium infecting monocyte/macrophages, downregulates TLR2 and 4 expression and LPS-induced activation of p38, ERK1/2 and NF-κB (Lin and Rikihisa, 2004). Also ReA-triggering bacteria produce numerous virulence factors targeted against MAPK pathways (Reviewed in Brodsky and Medzhitov, 2009). For example *Salmonella* TTSS effector SpvC inactivates p38 irreversibly by removing the phosphate from phosphothreonine in the activation loop (Zhu et al., 2007).

p38 isoforms are found both in cytoplasm and nucleus. Upon activation by stress stimuli, higher amounts of p38 relocate to the nucleus (Reviewed in Cargnello and Roux, 2011). However, some p38 substrates e.g. MAPKAPK2, 3 and 5 act as cytoplasmic anchors. Activation of p38-MAPKAPK2-complex leads to complex translocation to the cytoplasm (Ben-Levy et al., 1998, Gaestel, 2006). Cytoplasmic substrate can only be reached after complete activation and translocation (Gaestel, 2006). MAPKAPK2 and 3 control the gene expression also at the post-transcriptional level as they phosphorylate the ARE-binding mRNA stabilizing proteins like HuR (Reviewed in Cuadrado and Nebreda, 2010). MAPKAPK2 is essential for LPS-induced

cytokine synthesis as MAPKAPK2 knockout mice produce 90% less TNFα and the p38 pathway stabilizes stress-induced IL-6 and IL-8 mRNA expression through a MAPKAP2-dependent, ARE-targeted mechanism (Kotlyarov et al., 1999, Winzen et al., 1999). In a murine model of a collagen-induced arthritis, MAPKAPK2 deficient mice show increased resistance against arthritis (Hegen et al., 2006).

Among the most important functions of p38 is the regulation of cytokine production by creating a regulatory loop (Figure 5.). It has also been suggested that the inflammatory effects are mainly mediated by p38α isoform, as inhibition of p38α is sufficient for anti-inflammatory response in an *in vivo* mouse model (O'Keefe et al., 2007). p38 can modulate cytokine expression both by regulating transcription factors and mRNA stability and translation. p38α is the main isoform mediating the cytokine production (Reviewed in Cuenda and Rousseau, 2007). The p38 cascade can be activated by various cytokines including type I interferons like IFNγ. In human fibroblasts, IL-17 induced signaling functions through p38 and ERK1/2 activating AP-1, NF-κB and C/EBPβ (Cortez et al., 2007) and moreover, the signal transducer and activator of transcription 1 (STAT1) activation is partially regulated via p38 (Kovarik et al., 1999). p38 activity induces for example TNFα, IL-1, IL-6, IL-8 and cyclooxygenase-2 production and secretion especially in monocytes and macrophages (Reviewed in Feng and Li, 2011). p38 is known to activate TNFα expression in LPS-stimulated Schwann cells by regulating transcription (Cheng et al., 2007). IL-10 is among the most important anti-inflammatory cytokines. LPS-stimulated IL-10 production is dependent on p38 and JNK activity in human PBMC (Dobreva et al., 2009). LPS induces similar *IL10* gene transcription in human monocytes than in PBMCs but amount of produced IL-10 is lower (Dobreva et al., 2009). This might be explained by posttranscriptional regulation by different mRNA destabilizing 3'UTR motifs varying by cell type and activated by different signals (Dobreva et al., 2009, Powell et al., 2000). However, it has been shown that in macrophages, p38 mediated IL-10 production increases but IL-12 production decreases in response to Group B *Streptococcus* (GBS) infection (Bebien et al., 2012). p38 is activated by pore-forming toxin, βh/c, that is a major virulence factor for GBS. Thus the host response is modified in favor of the infecting bacteria.

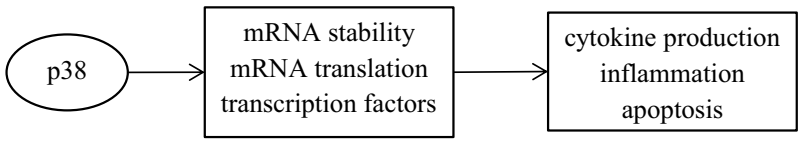

Figure 5. Examples of p38-mediated cellular functions.

In U937 monocytic cells, p38 mediates apoptosis through tumor suppressor p53 and the proapoptotic factor caspase-3 (Huh et al., 2004). ER stress induces p38 phosphorylation and induces autophagy and apoptosis through it in human fibroblasts (Kim et al., 2010). However, p38β, but not p38α, is essential to protect the rat mesangial smooth muscle cells from TNFα toxicity (Guo et al., 2001). In murine fibroblast cell line models, the TNFα-induced p38 activation increases NF-κB

activation promoting the cell survival signals rather than cell death (Lüschen et al., 2004). Moreover, ER stress induced CHOP regulates apoptosis. CHOP has two phosphorylation sites activated by p38 suggesting that p38 increases the activity of the CHOP transactivation domain (Reviewed in Darling and Cook, 2014) and is linked to maximal cell death response (Maytin et al., 2001). Thus, p38 isoforms may have different physiological functions or p38 may have dual role in regulating apoptosis.

2.7.1.4 Other mitogen-activated protein kinases

JNK1-3 (also called MAPK8-10, Table 4.) encoded by three genes are 85 % identical to each other, but several splicing variants exist (Reviewed in Cargnello and Roux, 2011 and Roux and Blenis, 2004). JNKs regulate stress responses in all tissues, but especially in neural tissue, and they also regulate inflammation and apoptosis. The JNK cascade is activated by environmental stress and growth factors or in response of protein synthesis inhibition (Reviewed in Johnson and Lapadat, 2002 and Qi and Elion, 2005). JNK is activated through phosphorylation of Thr-Pro-Tyr domain (Huang et al., 2009). All JNKs are important gene expression regulators as they phosphorylate the component of AP-1 transcription complex, the DNA binding protein c-Jun, increasing its transcriptional activity (Reviewed in Johnson and Lapadat, 2002). Moreover, it has been shown in a rat model that JNK-dependent AP-1 activation is a mediator of joint destruction in arthritis (Han et al., 2001). Thus JNK inhibitors may be of importance in developing treatment for arthritis (Han et al., 2001).

ERK1 (also called MAPK3, Table 4) is the first MAPK molecule identified. ERK1 and ERK2 (MAPK1) are 83% homologous by amino acid structure but alternatively spliced isoforms (ERK1b, ERK1c and ERK2b) exist. Both ERK1 and ERK2 are expressed in all tissues but higher levels are found in brain, skeletal muscle, thymus and heart (Reviewed in Cargnello and Roux, 2011). The ERK1/2 cascade regulates proliferation, differentiation, meiosis and in nerve cells, learning and memory (Reviewed in Qi and Elion, 2005). ERK is activated through Thr-Gln-Tyr domain (Huang et al., 2009) by growth factors, hormones like insulin, cytokines and osmotic stress. Activated ERK1 and 2 activate MAPKAPKs (RSKs, MNKs and MSKs), and other substrates e.g. STAT3 and ribosomal protein kinases (Reviewed in Cargnello and Roux, 2011 and Roux and Blenis, 2004). ERK1/2 is even able to bind directly to DNA and thus directly regulate IFNγ-induced genes (Hu et al., 2009).

ERK5 (MAPK7; big MK, BMK) is 51 % homologous to ERK2 but it is substantially larger than other MAPKs (approximately 110 kDa) (Reviewed in Plotnikov et al., 2011). Three splicing variants, ERK5a, b and c are known and it is expressed in all tissues. ERK5 cascade is less studied than other MAPK cascades, but it is known to regulate cell proliferation and differentiation, angiogenesis, cell survival and stress responses. ERK5 is activated by growth factors, cytokines e.g. IL-6, hyperosmolarity and oxidative stress (Reviewed in Cargnello and Roux, 2011 and Nithianandarajah-Jones et al., 2012). ERK5 activates MAPKAPKs but can also act as a transcription factor, DNA binding is mediated by its unique non-catalytic region (Reviewed in Plotnikov et al., 2011).

2.7.1.5 Double-stranded RNA -dependent kinase PKR

PKR (also called IFN-induced, dsRNA-activated protein kinase, or eukaryotic translation initiation factor 2-α kinase 2 [EIF2AK2]) is ubiquitously expressed in low levels. PKR can be linked to two protein families: the dsRNA-binding proteins (DRBPs) and the eIF2α-kinases (Reviewed in Dabo and Meurs, 2012). The DRBPs are a family of eukaryotic, prokaryotic and viral-encoded products that share common DRB domain (DRBD) in which PKR has two DRB motifs (DRBMs) (Figure 6.) (Saunders and Barber, 2003). eIF2α-kinases contain Ser/Thr kinase domain located at the C terminus (Reviewed in Dabo and Meurs, 2012). There are also TRAF-binding motifs in the second DRBD and at the C terminus of PKR molecule through which PKR regulators such as PKR activator (PACT) may bind (Reviewed in Dabo and Meurs, 2012, Gil et al., 2004). PKR is mainly found in the cytoplasm but also present in the nucleus. PKR contains three nuclear export sequences (NESs) (Takizawa et al., 2000). In the cytoplasm, PKR interacts with ribosomes whereas in nucleus, it is mainly located in the nucleolus where ribosomes are assembled (Reviewed in Dabo and Meurs, 2012). In the nucleus, PKR is in phosphorylated form; a phenomenon that is linked to cellular stress and even some pathologies e.g. acute leukemia (Blalock et al., 2011, Reviewed in Dabo and Meurs, 2012).

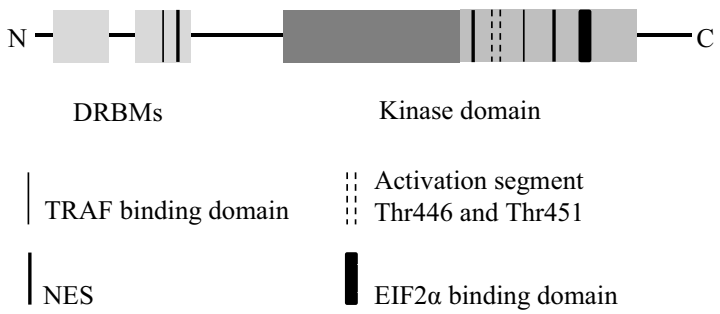

Figure 6. Ds RNA -dependent kinase (PKR) structure.

PKR was originally identified as a host defense factor against virus infections. The main function of PKR is to differentiate self and non-self as it recognizes the dsRNA of viral origin. It is a major mediator of the antiviral and antiproliferative activities of IFNs. Inactive PKR has disordered regions that reorganize when activated upon dsRNA binding (Reviewed in Nallagatla et al., 2011). Sufficiently long dsRNA, at least 33bp is needed in order to allow two PKR molecules (four DRBMs in total) to bind to the same RNA fragment and dimerize (Reviewed in Dabo and Meurs, 2012). After binding to the dsRNA, PKR conformation changes and dimerization through the kinase domain may take place.

PKR dimerization triggers autophosphorylation of Thr446 residue in the PKR activation loop. Autophosphorylation is required for the catalytic activity of PKR kinase domain and the substrate binding (Dey et al., 2005, Zhang et al., 2001). After Thr446 autophosphorylation, Thr451 can be consistently autophosphorylated (Zhang et

al., 2001). Activation of Thr446 and Thr451 are required for full kinase activity and moreover, kinase activity is dependent on Thr451 as mutation at that positions leads to inactive PKR molecule (Romano et al., 1998). The specificity of PKR as a Ser/Thr kinase is dependent on Thr451. After autophosphorylation and substrate binding, PKR mediates the transfer of phosphate from ATP to the acceptor site of the substrate (Reviewed in Dabo and Meurs, 2012). As PKR autophosphorylation may take place in the absence of dsRNA, there are other factors that may act as triggers (Lemaire et al., 2005). These PKR activators include proinflammatory stimuli, growth factors, cytokines, and oxidative stress (Reviewed in García et al., 2006). In addition, even certain cellular RNAs can activate PKR. The 3'UTRs mRNAs of some cytoskeletal proteins act as PKR activators and may be linked to the tumor suppressing properties of PKR (Davis and Watson, 1996). Moreover, INFγ mRNA translation is autoregulated by bindig to PKR (Cohen-Chalamish et al., 2009). INFγ mRNA forms a dsRNA by arranging three short helices aligned with the pseudoknot stem. Thus it can be hypothesized that PKR contributes to host defense against bacterial infections (Cabanski et al., 2008).

Activated PKR phosphorylates eIF2α (Figure 7.). PKR binds to α subunit of the eIF2 and prevents the binding of GTP-eIF2 to Met-tRNAi by inhibiting the regeneration of GDP to GTP (Reviewed in García et al., 2006, Majumdar and Maitra, 2005). The phosphorylation of eIF2α causes downregulation of the protein synthesis and the protein load in the ER is reduced. However, some RNAs coding stress related proteins may still undergo translation. RNAs that contain open reading frames (ORFs) in the 5'UTR can be translated into proteins that help the cells to recover from stress (Vattem and Wek, 2004). For example, activating transcription factor 4 (ATF4) is synthetized when proteins are misloaded or overloaded in the ER and general protein synthesis is disrupted. ATF4 then induces transcription of proapoptotic transcription factor CHOP, the ER chaperone BiP that reduces protein aggregation in the ER, and GADD34 that negatively regulates the stress-induced events leading to dephosphorylation of eIF2α (Liao et al., 2013, Vattem and Wek, 2004).

Mitochondrial UPR (mtUPR) is induced when unfolded proteins accumulate within the mitochondrial matrix. Many mtUPR responsive genes e.g. proteases and chaperones like mitochondrial chaperonin 60 (CPN60) are regulated through CHOP (Horibe and Hoogenraad, 2007). UPR in the ER and mitochondria differ from each other, even though both function through CHOP, since different stress proteins are upregulated (Aldridge et al., 2007). Moreover, mtUPR is dependent on PKR (Rath et al., 2012). Increased CPN60 expression is associated with increased PKR level in patients with inflammatory bowel disease and also in two murine models of colitis (Rath et al., 2012).

PKR is an important regulator of several transcriptional pathways, e.g. NF-κB and MAPK mediated pathways (Figure 7.). PKR activates NF-κB by forming a complex with IκB kinase (IKK) that leading to a degradation of NF-κB inhibitors IκBα and β (Zamanian-Daryoush et al., 2000). In response to stress signals, PKR acts as an activator of MAPK cascades and moreover, LPS and dsRNA-induced p38-mediated signaling requires PKR. PKR is required for p38 and MAPKAPK2 activation and it

regulates cytokine expression through activation of p38 and NF-κB in viral infection (Myskiw et al., 2009). Although the mechanism of the activation is not completely understood, PKR has been shown to interact with MAP2K6 leading to p38 activation. Viral infection by hepatitis C activates the p38-mediated activation of MSK2. MSK2 in turn binds to PKR stimulating its activation thus connecting the cellular stress signaling to the antiviral response (Kang and Ahn, 2011). It has also been suggested that PKR is a component of TLR4 signaling as it participates TIRAP- and MyD88-dependent signaling pathways (Horng et al., 2001). In murine fibroblasts, PKR is needed for full activation of p38 in response to LPS stimulation and p38-stimulated cytokine production is PKR-dependent (Goh et al., 2000).

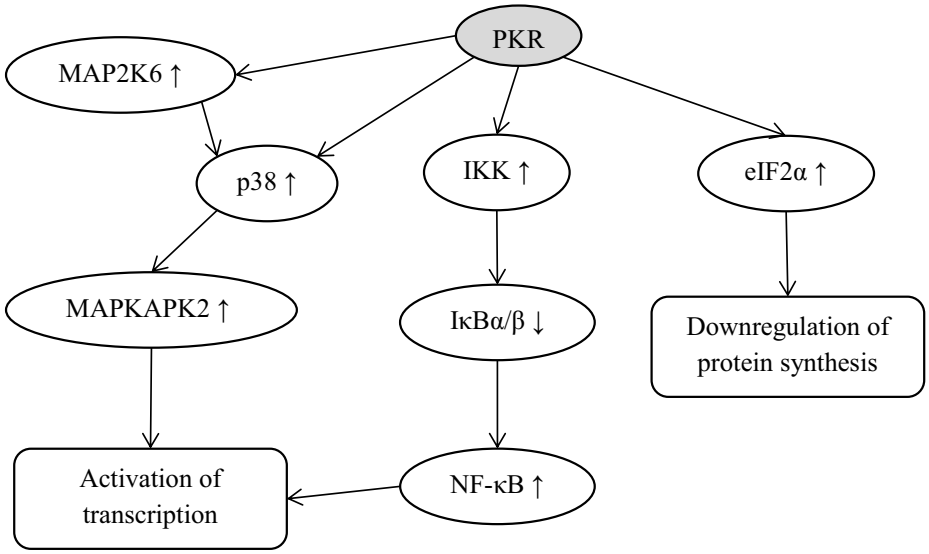

Figure 7. A schematic overview of ds RNA -dependent kinase (PKR)-regulated pathways. ↑ symbolizes activation and ↓ degradation

PKR induces TNFα expression in a p38-independent manner (Myskiw et al., 2009). After external stimuli, TNFα pre-mRNA splicing is regulated by PKR (Osman et al., 1999). PKR is also required to sustained TNFα response (Zamanian-Daryoush et al., 2000). However, TNFα-induced p38 activation is negatively regulated by PKR as PKR abrogation potentiates p38 phosphorylation in mouse embryonic fibroblasts (Takada et al., 2007). In murine alveolar macrophages, PKR abrogation impairs TNFα and IL-6 production but has no influence on p38 activation upon LPS stimulation (Cabanski et al., 2008) whereas PKR regulates JNK signaling by inhibiting MKK4 and c-Jun phosphorylation in response to LPS (Cabanski et al., 2008). The activation of p38 in human U937 monocytic cells by either TNFα or IFNγ was found to be PKR-independent. TNFα and IFNγ induced JNK activation was PKR-dependent whereas only IFNγ induced ERK activation in a PKR-dependent manner (Sharma et al., 2011). PKR is also involved in the production of anti-inflammatory cytokine IL-10 through JNK1 and NF-κB (Chakrabarti et al., 2008). In human monocytes, PKR is involved in

the regulation of IL-6, TNFα and IL-10 production in response to mycobacterial infection (Cheung et al., 2005). Interestingly, PKR activation promotes intracellular proliferation of *Leishmania* parasite in macrophages through induction of IL-10 production (Pereira et al., 2010). Thus PKR has a complex role in mediating the immune response.

In human U937 monocytic cells, TNFα-induced apoptosis is mediated by PKR (Yeung et al., 1996). Also in mouse fibroblasts, TNFα-induces NF-κB-regulated antiapoptotic protein expression through PKR (Takada et al., 2007). In macrophages, microbial-induced (e.g. *Salmonella*) apoptosis is dependent on TLR4 activation and is mediated by PKR induced protein synthesis block and IFN response factor 3 (Hsu et al., 2004). PKR mediates apoptosis possibly by eIF2α phosphorylation and upregulation of CHOP (Shimazawa and Hara, 2006). On the other hand, PKR may induce apoptosis by activating the ASK1-p38 MAPK/-JNK signaling pathways (Takizawa et al., 2002). Controversially, in mouse embryonic fibroblasts, PKR activity is not required to initiate apoptosis but delays cell death upon viral infection (Barry et al., 2009). This is explained by suggesting that PKR functions as a molecular clock chronologically activating both anti- and proapoptotic signaling pathways (Donzé et al., 2004). PKR first activates an NF-κB-dependent survival response and later induces eIF2α-mediated apoptosis (Donzé et al., 2004). These functions are mediated by two distinct mechanisms: NF-κB-activation by protein-protein interaction between PKR and IKKβ and eIF2α activation by the PKR kinase activity. NF-κB then induces the production of several survival proteins, e.g. c-IAPs which inhibit the proapoptotic caspases 3 and 7 whereas apoptotis is eventually triggered by the dsRNA (Donzé et al., 2004, Wang et al., 1998). Interestingly, the RNA stabilizing factor HuR is cleaved by caspases 3 and 7 in a PKR-dependent manner and these cleavage products (CPs) are linked to apoptosis (von Roretz and Gallouzi, 2010).

2.7.1.6 Kinase inhibitors

Protein kinases are widely studied using small, specific and cell-permeant inhibitors. Blocking the kinase function by using inhibitors is simple, relatively rapid and cost effective way to investigate the kinases and there is a wide range of commercially available inhibitors. Traditional p38 inhibitors e.g. SB203580 and SB202190 are ATP competing pyridinyl-imidazoles that occupy the adenine binding region of the ATP binding site. However, they only inhibit p38α and β isoforms, not γ or δ (Reviewed in Feng and Li, 2011), p38α being a predominant isoform in monocytes. Pyridynyl-imidazoles only inhibit the catalytic activity of p38, not the activation through phosphorylation. Since most of the kinases share conserved catalytic domain, the selectivity of these inhibitors can be questioned. It has been studied that SB203580 blocks 91 % and 87 % and SB202190 97% and 95 % of p38α and β kinase activity respectively when incubated together (Bain et al., 2007). The inhibitors also prevented the activation of other kinases such as CK1δ, RIP2 and GAK (Bain et al., 2007). DFG-pocket inhibitors e.g. BIRB-796, are more novel inhibitors and considered as more selective p38 inhibitors. They force a change in p38 conformation and hinder ATP from entering to the binding site (Pargellis et al., 2002). BIRB-796 inhibits all four p38

isoforms but also JNK2 (inhibition of 96, 87, 64 and 60 % of p38α, β, γ and δ, respectively and 96 % of JNK2) (Bain et al., 2007). Several p38 MAPK inhibitors have entered preclinical and clinical studies (Reviewed in Mayer and Callahan, 2006). However, despite the good anti-inflammatory effects in pre-clinical animal models, clinical p38 MAPK inhibitor studies have been discontinued mainly due to severe adverse effects caused by off-target effects (Reviewed in Mayer and Callahan, 2006).

2-aminopurine is shown to inhibit PKR autophosphorylation but it has been considered as a relatively unspecific compound (Posti et al., 1999). Thus an imidazole-oxindole structured ATP-competive PKR kinase inhibitor is widely used. Its selectivity towards PKR inhibition depends on the 3' substituted indolone core (Chen et al., 2008). It has been shown to inhibit RNA-induced PKR autophosphorylation and rescue PKR-dependent translation block (Jammi et al., 2003). Similar concerns as with the p38 kinase inhibitors have arosen regarding the specificity of the compound (Chen et al., 2008). p38 inhibitors have been under development as therapeutic agents applied to chronic inflammatory diseases, like RA (Reviewed in Fischer et al., 2011) but there is an increasing consensus that they are not suitable for such purposes due to severe adverse effects (Reviewed in Feng and Li, 2011 and Fischer et al., 2011). Rather the inhibitors might be of interest in other indications such as chronic obstructive pulmonary disease (Reviewed in Fischer et al., 2011). PKR inhibitors have not yet been regarded as potential drugs and there are no inhibitors under development for clinical applications (Bryk et al., 2011). However, there is an interest to develop better, more specific PKR inhibitors for research and clinical use (Bryk et al., 2011).

2.7.2 RNA-binding protein HuR

The regulation of the gene expression is an essential phenomenon by which the cell reacts to both internal and external challenges. RNA binding proteins (RBPs) are regulators of cellular signaling and cell fate as they control RNA metabolism. There are more than a thousand human RBPs and many of them have RNA-binding domains known as RNA recognition motifs (RRMs) (Reviewed in Hinman and Lou, 2008). During cellular stress e.g. infection, stress-sensitive genes controlled by RBPs are important. The production of these factors is ongoing while many other cellular activities are interrupted. Thus RBPs stabilizing AU-rich element (ARE)-containing mRNAs are also needed. In normal conditions, ARE-containing mRNAs are typically short-lived but in cellular stress they are stabilized by RBPs and translated into proteins (Reviewed in von Roretz et al., 2011).

RBP HuR belongs to Embryonic Lethal Abnormal Vision (ELAV) family of proteins and can also be referred as ELAV like protein 1 (ELAVL1) as Hu proteins share homology with the Drosophila ELAV protein. HuR is ubiquitously expressed 34 kDa protein that has three highly conserved RRMs (Figure 8.). RRM1 and 2 function together to bind to the ARE-containing mRNAs while RRM3 might bind to poly(A) tails or be involved in protein-protein interactions (Reviewed in Hinman and Lou, 2008). HuR is mainly located in the nucleus and is exported into the cytoplasm to function as mRNA stabilizer (Fan and Steitz, 1998). Nucleo-cytoplasmic shuttling is

considered as a critical step of mRNA stabilization by HuR (Reviewed in Doller et al., 2008). A hinge region between RRM2 and 3 has a nucleocytoplasmic shuttling sequence (HNS) that allows HuR to move between the cytoplasm and the nucleus as it contains both nuclear localization and nuclear export signal (Fan and Steitz, 1998). It also enhances the stability of the complex formed by HuR and its substrate (Fialcowitz-White et al., 2007).

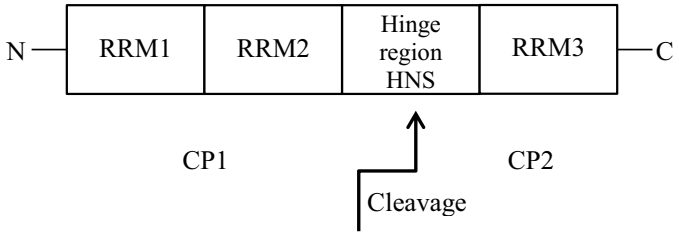

Figure 8. A schematic overview of human RNA binding protein (HuR) regions and cleavage site.

HuR is stimulated by various internal and external stimuli including cytokines, growth factors, inflammatory mediators, hormones, UV radiation, nutrient deprivation and oxidative stress (Reviewed in Doller et al., 2008). Activated MAPKs, including p38, may induce HuR export to the cytoplasm upon LPS stimulation (Lin et al., 2011). LPS-mediates p38 and JNK activation. MAPKs in turn regulate nucleocytoplasmic shuttling of HuR enhancing the mRNA stabilizing activity by direct interaction in smooth muscle cells (Lin et al., 2011). Anisomycin induces p38 activation that causes a rapid cytoplasmic accumulation of HuR in neuronal cells (Farooq et al., 2009). Also MAPKAPK2 is known to regulate HuR as MAPKAPK2 activation leads to the cytoplasmic accumulation of HuR (Tran et al., 2003).

HuR is an important mediator of the inflammatory and cellular stress responses. HuR enhances the protein translation of the target mRNA by binding to it, and thus stabilizing and protecting it from degradation (Reviewed in Hinman and Lou, 2008). One possible mechanism by which HuR functions is by forming oligomers on their target mRNAs and thereby blocking association of destabilizing RBPs (Fialcowitz-White et al., 2007). HuR stabilizes mRNA encoding cytokines, chemokines, growth factors, enzymes, tumor suppressors etc. (Figure 9.) (Eberhardt et al., 2007). HuR can act both as a promoter and a suppressor of inflammation (Katsanou et al., 2005). For example, HuR contributes to the inflammatory response by forming complexes with TNFα mRNA (Fialcowitz-White et al., 2007) and thus regulating the fate of TNFα independently from p38 pathway (Dean et al., 2001). In another study, it was suggested that TNFα mRNA translation requires HuR together with the p38 and MAPKAPK2 activity or inhibition of the ARE-binding and -destabilizing factor tristetraprolin (TTP) (Tiedje et al., 2012). TNFα in turn induces the HuR-dependent IL-6 expression through MAPKAPK2 activation (Xu et al., 2013) whereas IL-10, a potent anti-inflammatory cytokine, has been found to suppress p38 activation and HuR expression in human

U937 monocytic macrophages and mice leading to decreased TNFα production (Krishnamurthy et al., 2009, Rajasingh et al., 2006).

HuR can act both as pro- and antiapoptotic factor as in early state of stress, HuR inhibits apoptosis whereas in lethal stress, it promotes apoptosis (Figure 10.). This dual function is regulated by caspase-dependent cleavage. When HuR is located in the cytoplasm, it can be cleaved at a specific aspartate residue located in HNS region forming two CPs (Mazroui et al., 2008). HuR-CP1 (24 kDa) contains RRM1 and RRM2 and HuR-CP2 (8 kDa) contains RRM3. These CPs in turn promote apoptosis (Mazroui et al., 2008). HuR cleavage is mediated by caspases-3 and -7 and it has been shown that in HeLa cells, caspase-7 cleaves HuR but the proteolytic activity involves both caspase-7 and -3 (Mazroui et al., 2008). It has been suggested that the caspase-7/3 pathway is activated by caspase-8 that is in turn activated by PKR (von Roretz and Gallouzi, 2010). PKR, in an unphosphorylated form, enables Hur cleavage in HeLa cells and mouse fibroblasts (von Roretz and Gallouzi, 2010). Also ER stress promotes nuclear accumulation of HuR through CHOP leading to a promotion proapoptotic activities (Park et al., 2012). Studies with HuR knockout mice have revealed that HuR protects from pathological inflammation (Yiakouvaki et al., 2012) and, besides inflammation, HuR is involved in many human pathologies linked to dysregulated posttranscriptional regulation including cardiac diseases and cancer (Eberhardt et al., 2007).

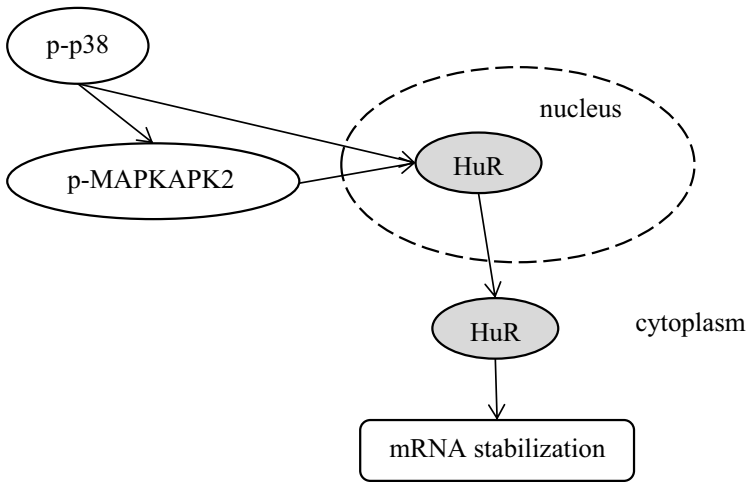

Figure 9. Phosphorylated p38 regulates human RNA binding protein (HuR) activity by inducing cytoplasmic accumulation.

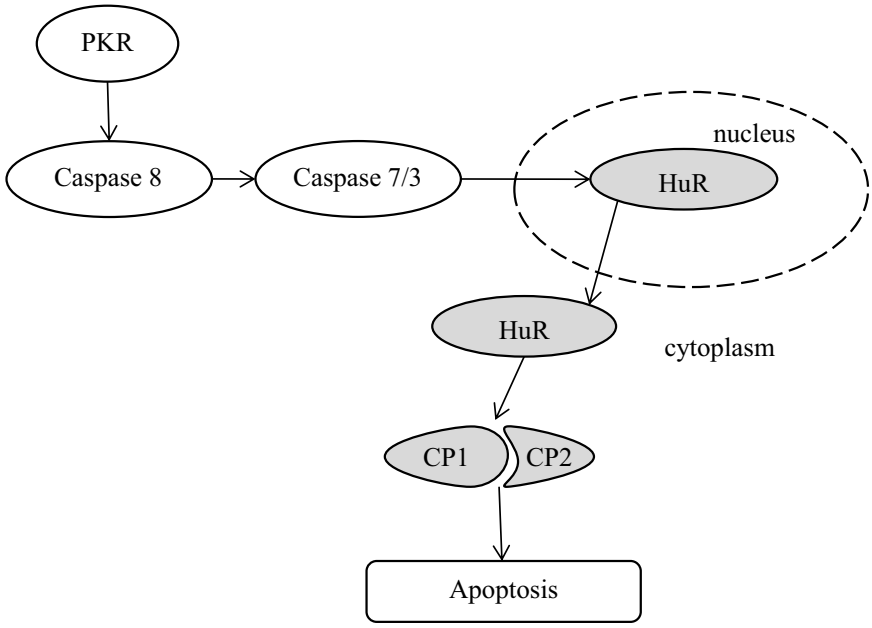

Figure 10. Ds RNA -dependent kinase (PKR) mediates human RNA binding protein (HuR) cleavage and apoptosis.

2.7.3 DNA-binding protein C/EBPβ

Transcription factors bind to specific DNA sequences in order to control transcription from DNA to mRNA. The transcription factor C/EBP family consists of six members (C/EBPα, β, γ, δ, ε and ζ) binding to the target gene promoter CCAAT box motif. These proteins regulate many functions, including metabolism, immunity and inflammatory response, cell cycle, proliferation and differentiation (Tsukada et al., 2011). C/EBP mouse homologue NF-IL6 is known to regulate intracellular *Salmonella* survival in mouse macrophages (Tanaka et al., 1995). C/EBPs contain DNA binding domain (DBD) and transcription activation domain (TAD), a basic leucine zipper domain (bZIP), that involves dimerization and DNA binding. DBD also acts as a nuclear localization signal (NLS) (Tsukada et al., 2011). Dimerization is essential for C/EBP activation and DNA binding. Besides homodimers, also heterodimers can be formed with C/EBP family members or other transcription factors including bZIP-structured transcription factors such as members of the Jun/Fos protein family, and non-bZIP proteins NF-κB and glucocorticoid receptor (Reviewed in Huber et al., 2012).

Alternative translation results in three C/EBPβ isoforms. Human liver-enriched activating protein star (LAP*) and LAP, also called C/EBPβ1 and C/EBPβ2, and LIP, also called C/EBPβ3, consist of 345, 322 and 147 amino acids, 44 kDa, 42 kDa and 20 kDa respectively. Mouse C/EBPβ sizes are LAP* 38 kDa, LAP 35 kDa and LIP 20 kDa (Reviewed in Huber et al., 2012). LAP* and LAP isoforms act as activators of gene transcription. In contrast, LIP is a transcription inhibitor as it does not contain

TAD1-3 like LAP* and LAP (Reviewed in Huber et al., 2012, Tsukada et al., 2011). Thus LAP/LIP ratio is of importance in the regulation of gene transcription. This ratio can be modified by involvement of translation initiation factors including eIF2α and CUG triplet repeat, RNA binding protein (CUGBP)-1. Moreover, C/EBPβ activity is dependent on the dimerization and interaction partners (Reviewed in Huber et al., 2012).

C/EBPβ is found in various tissues, e.g. liver, lung, kidneys and spleen. In monocyte/macrophages it is highly expressed and it is upregulated during monocytic differentiation (Natsuka et al., 1992). C/EBPβ is activated by various signals, e.g. cytokines, hormones and microbial products and compartments. Both C/EBPβ expression and activation are regulated by transcriptional and post-transcriptional mechanisms and protein-protein interactions. There are several binding sites for different transcription factors in the *C/EBPβ* promoter region. C/EBPβ mRNA transcription is regulated by C/EBPβ itself, signal transducer and activator of transcription 3 (STAT3), specificity protein 1 (Sp1), members of the bZIP-structured cAMP responsive element binding protein (CREB) and activating transcription factor (ATF) family among others. C/EBPβ may also interact with non-bZIP proteins such as NF-κB subunits p65 and p50 (Reviewed in Huber et al., 2012). The half-life of C/EBPβ mRNA is only approximately 40-60 min (Bergalet et al., 2011, Goethe and Phi-van, 1997) but it can be prolonged by RNA binding proteins (RBPs). The half-life of C/EBPβ isoforms varies, for LAP* and LAP it is approximately 2 h whereas for LIP it is 8.5 h (Reviewed in Huber et al., 2012). C/EBPβ is also a ligand for RBP HuR and C/EBPβ expression is increased by HuR in murine lymphoma cells (Bergalet et al., 2011). HuR prolongs the C/EBPβ mRNA half-life up to 110 min (Bergalet et al., 2011, Reviewed in Huber et al., 2012). However, controversial findings where HuR binding decreases the C/EBPβ expression exist, as HuR binding is suggested to lead to a nuclear retention of C/EBPβ mRNA in murine fibroblasts (Cherry et al., 2008). HuR also inhibits the cytostatic activity of C/EBPβ (Basu et al., 2011). C/EBPβ production and activity can be induced by LPS as well as other bacterial compartments and cytokines including IL-1, IL-6, TNFα and IFNγ (Reviewed in Huber et al., 2012). In human macrophages, an anti-inflammatory cytokine IL-10 upregulates LIP production via STAT3 (Tanaka et al., 2005). In murine macrophages, C/EBPβ activation upon LPS stimulation is dependent on p38 activity as C/EBPβ activity was found to be reduced in p38-deficient cells (Kang et al., 2008). In primary human monocytes, IFNγ-induced p38-dependent C/EBPβ phosphorylation was increased (Reviewed in Huber et al., 2012, Stoffels et al., 2006). In contrast, it has been shown that the inhibition of the p38 MAPK by a specific inhibitor SB202190 up-regulated C/EBPβ in human promyelocytic leukemia cells (Ji and Studzinski, 2004).

C/EBPβ regulates transcription of various genes; inflammatory cytokines, chemokines and receptors among others (Figure 11.). For example, cytokines such as TNFα, IL-6, IL-1, IL-8 and IL-10 and receptors like macrophage colony-stimulating factor receptor (M-CSFR) and IL-13RA1 are regulated by C/EBPβ (Reviewed in Huber et al., 2012, Su et al., 2003). C/EBPβ dimerization, localization, DNA binding capacity and activity is post-translationally regulated by phosphorylation, acetylation, methylation, sumoylation and proteolysis. Besides activator, C/EBPβ also functions as a repressor.

C/EBPβ interaction reduces the level of with NF-κB inhibitor IκBα in macrophages leading to induced NF-κB signaling (Cappello et al., 2009, Reviewed in Huber et al., 2012). Also tolerance against repeated TNFα stimulation in human monocytic cells is mediated by C/EBPβ (Weber et al., 2003). In TNFα tolerant monocytic cells, C/EBPβ prevents NF-κB subunit p65 phosphorylation and thus inhibits NF-κB-mediated transcription (Zwergal et al., 2006). Thus the role of C/EBPβ is complex. C/EBPβ and NF-κB complex generally activate promoters with C/EBPβ binding sites and inhibit promoters with NF-κB binding sites as C/EBPβ prevents the NF-κB interactions (Stein and Baldwin, 1993, Weber et al., 2003). Besides LIP, also other C/EBPβ isoforms may have inhibitory functions. This is supported by the finding that IL-12 is negatively regulated by LAP (Uematsu et al., 2007). C/EBPβ deficient mice have increased susceptibility to *Candida albicans*, *Listeria monocytogenes* and *Salmonella* Typhimurium infections and *S.* Typhimurium was detected to escape from the phagosomes to the cytosol (Screpanti et al., 1995, Tanaka et al., 1995) although LAP expression is not essential for elimination of intracellular bacteria (Uematsu et al., 2007). However, production of molecules important in antimicrobial activity, like NO synthase (iNOS), is C/EBPβ-dependent (Reviewed in Huber et al., 2012).

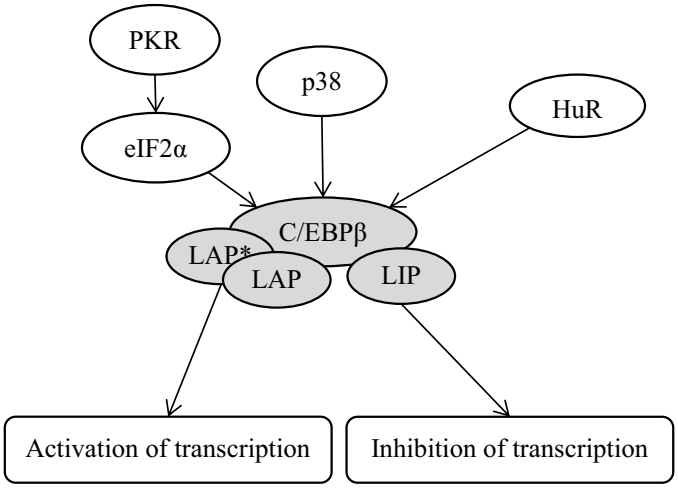

Figure 11. CCAAT enhancer binding protein beta (C/EBPβ), an activator (liver-enriched activator protein, LAP, and LAP*) and inhibitor (liver-enriched inhibitory protein, LIP) of transcription, is regulated by multiple factors.

3 AIMS OF THE STUDY

HLA-B27 is a well-known risk factor for the SpAs. Although HLA-B27 is extensively studied, it is not known how HLA-B27 contributes to the disease susceptibility. Previous results from our group indicate that HLA-B27 modulates the host-pathogen interaction between ReA triggering *Salmonella* and host monocytes. Thus the main object of this study was to clarify whether the expression of HLA-B27 alone regulates the expression and functionality of intracellular signaling molecules of monocytic macrophages enabling the persistence of bacteria or bacterial compartments.

Specific aims of the study were:

1. To identify MAP kinases important in the elimination of intracellular *Salmonella* in HLA-B27 expressing monocytic macrophages.
2. To study whether the expression of HLA-B27 has an effect on PKR, C/EBPβ and HuR that are involved in the immune response in monocytic macrophages.
3. To study whether the modulatory effects of HLA-B27 on these molecules are dependent on the misfolding feature of HLA-B27 heavy chains.

4 MATERIALS AND METHODS

4.1 Cells and cell culture

The human monocytic cell line U937 was obtained from American Type Culture Collection (ATCC) (Rockville, MD). The U937 cells express the HLA class I alleles A3, A26, B18, B51, Cw1, and Cw3 (Sundstrom and Nilsson, 1976). The cells (transfectants described below) were maintained in supplemented Roswell Park Memorial Institute (RPMI) 1640 medium. The medium was supplemented with 10% fetal calf or bovine serum (FCS, FBS; PAA Laboratories, Linz, Austria), 1.8 mM L-glutamine (Biological Industries, Kibbutz Beit Haemek, Israel), and 50 μg/ml of gentamicin (Biological Industries). The cells were kept at 37°C in a humidified athmosphere of 5% CO_2 (Laitio et al., 1997).

The U937 HLA-B27g (B27g) transfectant was created by electroporation of the full-length 6 kbp genomic clone of HLA–B*2705 genomic DNA in the pUC19 vector (kind gift from Dr. Joel D. Taurog) (Taurog et al., 1988). In order to confer resistance to Geneticin [G-418], the pSV2neo plasmid was cotransfected (Laitio et al., 1997). The U937 transfectants expressing mutated HLA–B*2705 HCs were constructed by site-directed mutagenesis (Altered Sites; Promega, Madison, WI) and cotransfection of mutated HLA–B*2705 genomic DNA in the pUC19 vector and pSV2neo by electroporation (Penttinen et al., 2004). Mutated transfectants B27.H9F, B27.E45M, and B27.C67A have 1 amino acid substitution (F for H at position 9, M for E at position 45, and A for C at position 67, respectively). B27.A2B contains 6 amino acid substitutions (H9F, T24A, E45M, I66K, C67V, and K70H) (Colbert et al., 1993, Dangoria et al., 2002, Mear et al., 1999). Complementary DNA (cDNA) of HLA–B*2705 (B27cDNA) cloned into the *Bam* HI site of the RSV5neo vector (kind gift from Dr. Beatrice Carreno) was introduced using liposomal transfection (Laitio et al., 1997). For mock transfections, cells were either transfected with pSV2neo or RSV2neo alone. New batches of the transfectants (stored at −135°C in Biofreezer) were introduced at least every three months. Transfectants were stable and new batches were always selected for G-418 resistance and surface expression of the transfected HLA molecule and screened to exclude mycoplasma infection (Laitio et al., 1997, Penttinen et al., 2004).

4.2 Isolation of peripheral blood monocytes

Human peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of healthy blood donors (Finnish Red Cross, Helsinki, Finland). The isolation was performed by Ficoll-Paque gradient centrifugation (GE Healthcare, Uppsala, Sweden) (Wuorela et al., 1993). Monocytes were allowed to adhere to 24-well tissue culture plates (1.9 cm^2; Greiner, Frickenhausen, Germany) in the presence of 10% heat-inactivated FCS for 1 hour at 37°C in a humidified atmosphere of 5% CO_2. Non-

adherent cells were removed from the plates by washing 3 times with Hanks' balanced salt solution (HBSS).

4.3 Bacterial strains

Salmonella Enteritidis strain used was a stool isolate from a patient with *Salmonella*-triggered ReA (Laitio et al., 1997). The *S.* Enteritidis strain was also used for green fluorescent protein (GFP) transformation (Penttinen et al., 2004). Briefly, pACYC plasmid containing GFP DNA was transfected to *S.* Enteritidis by electroporation. *Salmonella* was grown in 10 ml Luria-Bertani (LB) broth at 37°C for 18 hours. The logarithmic phase of growth was obtained by transferring 500 µl of the bacterial culture into 10 ml of fresh LB broth for a further 2 hours (Laitio et al., 1997). GFP-transformed bacteria were grown as described above, but in 20 µg/ml chloramphenicol supplemented LB medium in order to maintain the *GFP* gene–containing vector.

4.4 Exposure of cells to bacteria and LPS

The cells were diluted to a concentration of 1.0×10^6/ml and seeded into tissue culture flasks (25 cm^2; Greiner, Frickenhausen, Germany) or 24-well tissue culture plates in supplemented RPMI 1640 (described in Chapter 4.1) containing 10 ng/ml of phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, St. Louis, MO) for 22–24 hours at 37°C in a humidified atmosphere of 5% CO_2 to differentiate them toward mature, adherent macrophages. Two hours prior to the bacterial infection, adherent cells were washed with HBSS, and fresh, prewarmed RPMI 1640 supplemented with 10% human AB serum (Finnish Red Cross, Helsinki, Finland) was added to the cells. After incubation, the cells were cocultured with *S.* Enteritidis at a 1:5 to 10 cell-bacteria ratio for 1 hour at 37°C. The cells were then washed 3 times with HBSS and incubated at 37°C in supplemented RPMI 1640 containing gentamicin to kill the remaining extracellular bacteria until further experiments were performed.

For the LPS stimulation, the cells were diluted into culture flasks and stimulated with PMA as described above. After 22-24 h of incubation, cells were stimulated with 500 ng/ml of *S.* Enteritidis LPS. The cells were then incubated at 37°C until further experiments were performed.

4.5 Measurement of the number of viable intracellular bacteria

The number of viable intracellular bacteria was determined using colony-forming unit (CFU) method (Laitio et al., 1997). After defined periods of time (counted from the removal of the extracellular bacteria not bound to the cells, by washing at 1 hour after infection), the cells were harvested by scraping and the number of living cells was counted after staining with Trypan Blue. To release the intracellular bacteria, the cells were lysed with 1% Triton X-100 (after washing, the cells were kept in supplemented RPMI 1640 containing gentamicin in order to kill the remaining extracellular

bacteria.). The bacteria were resuspended to phosphate-buffered saline (PBS), a dilution series was prepared and the dilutions were cultured in LB plates at 37°C overnight.

4.6 Inhibition assays

The inhibitors were added to the U937 transfectants or PBMCs 1-2 hours after bacterial infection or 15, 30, 60 90 or 120 minutes prior to LPS stimulation. Inhibitors were used at 1, 4, 5, 7, 10 or 50 μ*M* concentrations depending on the inhibitor or experiment. Inhibitors used were p38 MAPK inhibitors SB203580 (Promega), SB202190 (BioSource, Camarillo, CA), SC68376 (Calbiochem, Darmstadt, Germany) and BIRB 796 (Calbiochem), PKR inhibitor PKR+ (Calbiochem), MEK inhibitor PD98059 (Calbiochem), the NF-κB inhibitor sulphasalazine (Sigma-Aldrich), the reversible inhibitor of JNK SP600125 (Calbiochem), Raf1 kinase inhibitor I GW5074 (Calbiochem), Casein kinase I inhibitor V (Calbiochem) and Src kinase inhibitor I (Calbiochem). In addition, 10 ng/ml of recombinant human IL-10 (R&D Systems, Minneapolis, MN) was used. SB202474 (Calbiochem) was used as a negative control for p38 inhibition, and PKR- as a negative control for PKR inhibition (Calbiochem).

4.7 Transfection with siRNA

The cells were diluted to a concentration of 0.5×10^6/ml, seeded into 24-well tissue culture plates, and incubated with supplemented RPMI 1640 and 10 ng/ml PMA for 24 hours at 37°C in a humidified atmosphere of 5% CO_2. A SignalSilence Pool p38 MAPK siRNA kit (Cell Signaling Technology, Danvers, MA) and PKR siRNA (Santa Cruz Biotechnology Inc.) were used according to the manufacturers' instructions (final concentration of siRNA was 10 n*M*) The 24-well tissue culture plates were centrifuged at 1,500*g* for 5 minutes at 20°C to bring the siRNA into contact with the cells. After 48 hours of incubation at 37°C, the cells were infected as described above. The efficiency of siRNA transfection was determined by Western blotting. The number of living and dead cells was counted after staining with Trypan blue and the quantity of intracellular bacteria was determined with CFU-method.

4.8 Immunofluorescence and flow cytometry

The cell surface expression of the transfected HLA molecules was confirmed by immunofluorescence and flow cytometry (BD Immunocytometry Systems, San Jose, CA) (Laitio et al., 1997, Penttinen et al., 2004). Briefly, the cells were stained with anti-human HLA-B27 monoclonal antibody (mAb) (clone FD705-9EIEI0; One Lambda, Canoga Park, CA) and with fluorescein isothiocyanate-conjugated secondary antibody. mAb recognizing chicken T cells (3G6) was used as a subclass-matched negative control.

To detect intracellular proteins, PMA-maturated and LPS-stimulated cells were collected at indicated time points and washed with PBS or tris-buffered saline (TBS). The cells were fixed with 1.5% formaldehyde for 10 minutes at room temperature

(RT), and then permeabilized with 100% ice-cold methanol for 10 minutes. The samples were stored at −20°C in 100% methanol until staining. For staining, the cells were divided into tubes (500 000 cells/tube) and washed twice with PBS or TBS supplemented with 1% BSA (staining buffer). Anti-C/EBPβ (C-19) (sc-150; Santa Cruz Biotechnology Inc., Santa Cruz, CA) and anti-HuR (6A97; Santa Cruz Biotechnology Inc.) antibodies were added on cells (1:50, 100 µl/tube) and incubated 30 minutes in dark at RT. The cells were washed three times with staining buffer. FITC-conjugated secondary antibody was added (1:200, 100 µl/tube) and incubated 30 minutes at RT in dark. Cells were washed three times with PBS or TBS and 500 µl of PBS or TBS containing 1% formaldehyde or 500 µl of EPICS-PBS/TBS (145 mM NaCl, 18 mM K_2HPO_4, 10 mM KH_2PO_4) was added to each tube. The samples were analysed by FACSCalibur flow cytometry (Becton Dickinson Immunocytometry Systems, San Jose, CA). The results are shown as a fold induction of relative amount of positive cells or fluorescence intensity where the value obtained at 0-h time point was normalized to one, and the values at 5-h time point are proportional to that.

4.9 Fluorescence and confocal microscopy

For fluorescence microscopy, 13-mm round coverslips (Sarstedt, Newton, NC) were placed onto the 24-well tissue culture plates. Cells were diluted to a concentration of 2 x 10^5/ml and seeded into the plates. The cells were infected with GFP-transformed *S.* Enteritidis as described earlier (Chapters 4.3 and 4.4). At 24 hours after infection, the cells were examined with a Leitz Dialux 20 fluorescence microscope (Leica Microsystems, Wetzlar, Germany) using a 63x 1.4 numerical aperture oil-immersion Fluotar objective.

For confocal microscopy, the cells were seeded into tissue culture flasks as described earlier (Chapter 4.4). The cells (7.5×10^4) were centrifuged on microscope slides, air-dried, and mounted with Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL). The cells were visualized with an LSM 510 META laser scanning microscope (Carl Zeiss, Jena, Germany) using a 63x 1.4 numerical aperture oil-immersion plan apochromat objective equipped with differential interference contrast optics. Confocal images were collected using 488-nm excitation light from a 4-line argon laser and a 500–550 nm band-pass filter.

4.10 Preparation of cell extracts

The cells were harvested by scraping at the indicated time points, washed two times with ice-cold PBS and frozen immediately at −70°C. Samples were resuspended with lysis buffer C (420 mm NaCl; 25% glycerol; 0.2 mm EDTA; 1.5 mm $MgCl_2$; 20 mm HEPES pH 7.9), supplemented with Complete Mini Protease inhibitor cocktail tablet (Roche Diagnostics, Mannheim, Germany) and phosSTOP phosphatase inhibitor tablet (Roche). The samples were incubated for 60 minutes on ice at +4°C and centrifuged 20 minutes at 12 000 *g* at +4°C. Supernatants were collected as whole-cell extracts containing soluble proteins. The protein concentration was measured by Bradford protein assay (Bio-Rad, Hercules, CA).

4.11 Gel electrophoresis and Western blot analysis

Whole-cell extracts (containing 30 μg of protein) in Laemmli buffer were separated on a 10% sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane (Protran Nitrocellulose; Schleicher & Schuell, Keene, NH) by semidry transfer apparatus (Bio-Rad). The blots were analysed by enhanced chemiluminescence method (ECL; Millipore, Billerica, MA, USA). The following antibodies were used: anti-p38 (p38α/β) 1:1000 (Cell Signaling Technology, Danvers, MA, USA), anti-C/EBPβ (C-19) 1:400 (sc-150; Santa Cruz Biotechnology Inc.), anti-phospho-PKR (Thr 451) 1:1000 (Upstate, Lake Placid, NY, USA), anti-PKR 1:1000 (Cell Signaling Technology), anti-HuR (6A97) 1:400 (sc-71290, Santa Cruz Biotechnology Inc.), anti-Hsc70 for HSPA8 1:10 000 (Stressgen Bioreagents, Ann Arbor, MI, USA), monoclonal anti-rabbit (HRP) 1:10 000 (Dako, Glostrup, Denmark), anti-mouse (HRP) 1:10 000 (Dako) and anti-rat (HRP) 1:10 000 (Stressgen). Antibody neutralization peptide for C/EBPβ (C-19) antibody (Santa Cruz Biotechnolocy Inc.) was used according to manufacturer's instructions. Densitometric analysis was performed with MCID 5+ image analysis software (InterFocus Imaging Ltd, Cambridge, UK). Intensity of each band was determined and proportioned to a loading control. The results are shown as a fold induction of relative intensity (RI) where the intensity of selected band was given value one, and the others are proportional to that.

4.12 Quantitation of TNFα and IL-10 secretion

To measure TNFα and IL-10 production with the enzyme-linked immunosorbent assay (ELISA), the cells were seeded into 24-well tissue culture-plate and treated as described earlier. After 22–24 hour of incubation, the medium was removed and fresh supplemented RPMI 1640 was added to the cells. The cells were then treated with inhibitors (described at Chapter 4.6) and after 15 minutes of incubation, stimulated with 500 ng/ml LPS. The cell free supernatants were collected after 6 h and 24 h incubation and frozen immediately −70°C before use. TNFα and IL-10 concentrations in the culture media were measured by sandwich-ELISA method. Commercially available antibody pair Mab1 and Mab11 (551220 and 554511; BD Biosciences, San Jose, CA) was used to detect TNFα and JES3-9D7 with JES3-12G8 to detect IL-10 (554497 and 554499; BD Biosciences). Recombinant human TNFα and IL-10 (R&D Systems Inc., Minneapolis, MN) were used as a standard. Streptavidin–horseradish peroxidase (HRP) conjugate was purchased from Invitrogen and tetramethylbenzidine (TMB) substrate from Sigma-Aldrich. The absorbance was measured at a wavelength of 450 nm.

4.13 Statistical analysis.

Statistical comparison of the data was performed with Student's paired two-tailed *t*-test, Wilcoxon matched pairs Signed-Rank test and Wilcoxon Rank-Sum test.

5 RESULTS

5.1 Intracellular bacterial replication in human monocytic macrophages (I)

In this study, the relevance of several intracellular signaling pathways contributing to host-microbe interaction was elucidated. The studies revealed that p38 MAPK activity was an important factor in host-pathogen interaction and it regulated the replication of *Salmonella* in U937 monocytic macrophages. U937 cells were able to eliminate the intracellular bacteria. However, after inhibition with a specific, commercially available p38 inhibitor, the bacteria were able to survive and replicate intracellularly. Interestingly, SP600125 and PD98059 used as inhibitors of JNK and Erk1/2 MAP kinases and sulphasalatazine used as a NF-κB inhibitor did not affect the replication of *Salmonella* suggesting that JNK and Erk1/2 MAP (MEK) kinases or NF-kB are not involved in the regulation of intracellular *Salmonella*.

5.1.1 p38 MAPK-related signaling was dysregulated in HLA-B27 expressing cells

To compare the effect of p38 MAPK inhibition caused by SB203580 in different HLA-B27 expressing U937 transfectants, cells were infected, and the intracellular fate of *S.* Enteritidis was studied. Shortly after infection, all U937 transfectants contain similar amounts of intracellular bacteria. In addition, a decrease in the number of intracellular *S.* Enteritidis was detected 8 hours after infection in all HLA-B27 transfectants and vector transfected mock control (I: Figure 4A, B). The cell lines expressing misfolding and homodimerizing forms of HLA-B27 HCs (B27g, B27cDNA and B27.H9F mutant transfectant) contained increased number of intracellular bacteria 20 hours after infection whereas other cells (mock and mutant transfectants B27.A2B, B27.E45M and B27.C67A) were able to eliminate the bacteria or limit the bacterial growth more efficiently (I: Figure 4A). Interestingly, p38 inhibition with SB203580 had a dramatic effect on the survival and replication of *S.* Enteritidis in mock (a 10-fold increase in the number of bacteria) and in transfectants expressing HLA-B27 HCs with a lower tendency to misfold and/or form homodimers (B27.A2B, B27.E45M and B27.C67A; 6-, 21-, 15-fold, respectively), as a clear increase in the number of intracellular bacteria was observed (I: Figure 4B). In contrast, in the cells expressing the misfolding and homodimerizing forms of HLA-B27 HCs (B27g, B27cDNA, and B27.H9F), p38 inhibition with SB203580 had no notable effect (I: Figure 4A, B). However, the p38 inhibition induces a similar bacterial survival in cells expressing normally folding HLA-B27 HCs or in mock than was observed in misfolding HCs expressing transfectants without any inhibition (I: Figure 4A, B, Figure 5). Since the behavior of B27.A2B, B27.E45M, and B27.C67A cells was similar to the behavior of the mock-transfected control cells, it is plausible that the misfolding and/or homodimerization features of HLA-B27 HCs are at least partially responsible for the impaired ability of HLA-B27-expressing cells to resist intracellular replication of *S.* Enteritidis (Figure 12).

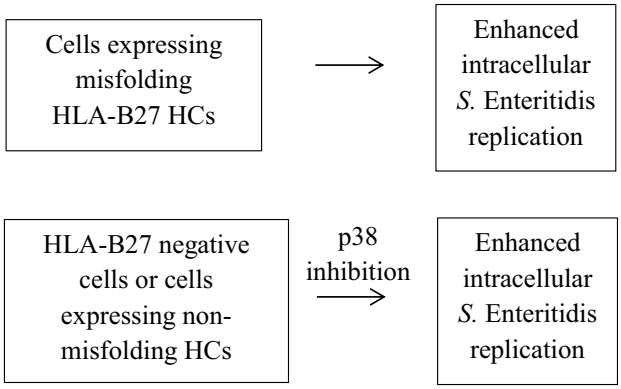

Figure 12. Intracellular *S.* Enteritidis is able to replicate within U937 cells expressing misfolding HLA-B27 heavy chains (HCs). Similar replication is seen in HLA-B27 negative cells and in cells expressing non-misfolding forms of HLA-B27 HCs after p38 inhibition.

When GFP-transformed *S.* Enteritidis-infected cells were examined microscopically, it was revealed that not all of the cells contained intracellular bacteria. However, among the infected cells, the number of cells containing multiple bacteria was remarkably higher in B27g- and B27cDNA-expressing cells that contain misfolding HLA-B27 HCs than in control cells or cells expressing non-misfolding HLA-B27 HCs (mock, B27.A2B, B27.C67A, and B27.E45M, I: Table1). In the absence of p38 inhibition by SB203580, these cells typically contained only one bacterium per cell. In contrast, upon treatment with SB203580, the number of cells containing multiple bacteria increased. The effect of SB203580 treatment was dose-dependent, but even the highest concentration did not induce cell death.

SB203580 is a relatively specific p38 inhibitor and its effect on *Salmonella* survival is dose-dependent (I: Figure 1C). In order to further examine the specificity of p38 inhibition, experiments with two other p38 inhibitors were performed (I: Figure 2A). SB202190 has a similar chemical structure than SB203580. SB202474, a structurally similar compound to SB203580 and SB202190 that lacks the ability to inhibit p38, was used as a negative control for p38 inhibition. The structure of SC68376 differs from the other inhibitors used in this study. SB202190 induced even higher increase (15-fold) in the amount of intracellular bacteria than SB203580 (10-fold, I: Figure 2A). SB202474 also induced a smaller increase (5-fold) indicating that in addition to p38, other molecules effect on intracellular *Salmonella* replication and are inhibited by the p38 inhibitors SB2023580 and SB202190. Nevertheless, these results indicate that p38 is indeed involved, since SB203580 and SB202190 had a profound effect compared with lesser effect caused by SB202474. In addition, a statistically significant small increase (2-fold) in the number of intracellular *Salmonella* was detected in cells treated with SC68376.To further clarify whether the effect observed with the p38 inhibitors was really caused by the inhibition of p38 rather than unspecific inhibition of other molecules experiments with p38 siRNA were performed (I: Figure 2B, C). In p38 siRNA–transfected mock transfectants, *S.* Enteritidis replication was substantially increased (3-fold) in comparison with the nontransfected counterparts.

To investigate whether p38 activation is relevant in *Salmonella* survival in human primary monocytic cells, peripheral blood monocytes were obtained from six individuals. Isolated monocytes were *S.* Enteritidis-infected and treated with inhibitors of p38 activation. When SB202190 was used, the number of intracellular bacteria increased in every batch of monocytes (1,5-3-fold, I: Figure 3). One batch of monocytes did not react to the treatment with SB203580 or SC68376. Altogether, SB203580 and SC68376 induced increase in the number of bacteria in 5 of 6 batches and 4 of 6 batches, respectively. In contrast, SB202474 induced intracellular bacterial growth only in 2 of the 6 batches of monocytes. However, the statistical significance was not obtained.

5.1.2 Inhibition of other signaling molecules

JNK signaling pathway was inhibited with the inhibitor SP600125, Erk1/2 pathway with the MEK-inhibitor PD98059, oxygen radical –dependent activation of NF-κB with sulphasalazine and cytokine synthesis with human recombinant IL-10 (I: Figure 1A, B). None of these inhibitors had a significant effect on intracellular *S.* Enteritidis survival or replication as the bacteria were eliminated from the inhibitor treated U937 mock cells similarly to untreated control cells.

5.2 Increased expression but impaired phosphorylation of PKR in HLA-B27 expressing cells (II)

After *S.* Enteritidis infection or stimulation with *S.* Enteritidis LPS, increased PKR expression was observed in PMA-maturated B27g U937 transfectants compared with the mock transfected cells (II: Figure 1A, B). The cells expressing mutated HLA-B27 HCs that continue to misfold (B27.H9F) also had increased PKR expression level whereas cells expressing HCs with lower tendency to misfold (B27.E45M) had similar PKR expression level than mock (II: Figure 1A, B). Moreover, even before any stimulation other than PMA, B27g cells had higher PKR expression level (II: Figure 1B). These results provided evidence that PKR expression is linked to the misfolding of HLA-B27 HCs (Figure 13).

The transfectants that expressed misfolded HLA-B27 HCs and had increased PKR expression level (B27g and B27.H9F), unexpectedly had lower PKR phosphorylation level (II: Figure 2A, B). Higher amount of phospho-PKR was consistently observed in cells expressing HCs with a tendency to fold correctly (B27.E45M and mock) both after *Salmonella*-infection and LPS stimulation (II: Figure 2A, B). More phosphorylated PKR was detected after infection with *Salmonella* than LPS stimulation. This might be explained by differences between infection and stimulation protocols. After infection, the collected samples contained only adherent cells whereas after stimulation, also non-adherent cells were collected.

Overall, the inhibition of the PKR activity had a minor effect on PKR expression (II: Figure 4B). However, in LPS-stimulated B27g cells incubated with the PKR inhibitor (PKR+), PKR expression was decreased in comparison with untreated cells. A smaller

effect was observed in LPS-stimulated mock transfectants (II: Figure 4B). In LPS-treated mock cells, phosphorylation of PKR was decreased upon PKR inhibition (II: Figure 4A). Additional experiments were performed using p38 MAPK inhibitors, and no significant effect on either PKR expression or activation was detected in any of the transfectants studied (data not shown).

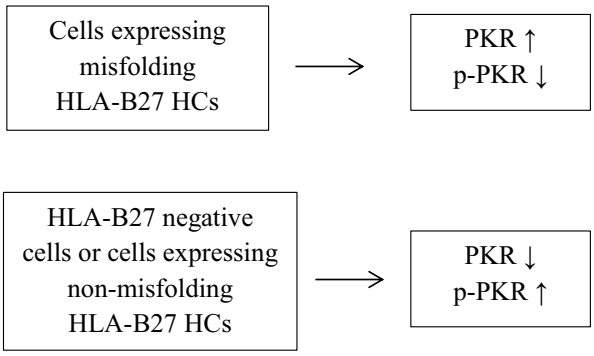

Figure 13. Ds RNA -dependent kinase (PKR) expression is enhanced but phosphorylation is impaired in U937 cells expressing misfolding HLA-B27 heavy chains (HCs) compared with cells expressing non-misfolding HLA-B27 HCs.

5.3 Increased expression of C/EBPβ isoforms in HLA-B27 expressing cells (II)

Relatively similar levels of C/EBPβ isoforms (LAP* and LAP 44 and 42 kDa, LIP 20 kDa) were expressed in all transfectants shortly after *Salmonella* infection (II: Figure 3A). However, after incubation, C/EBPβ expression was increased in B27g and B27.H9F transfectants (Figure 14.). Only a modest increase was observed in E45M, and no increase at all in mock. Similar results were obtained when transfectants were stimulated with *S.* Enteritidis LPS (II: Figure 3B, Figure 5C, D). Thus, the expression of C/EBPβ isoforms was increased in all transfectants expressing HLA-B27 and the more dramatic increase was observed in cells expressing misfolding HLA-B27.

When C/EBPβ expression was studied by Western blot, several bands were detected. Thus the specificity of C/EBPβ antibody was also confirmed with a specific neutralizing peptide blocking the binding site of the antibody (Staiger et al., 2009). After blocking with the peptide, no C/EBPβ was detected indicating that all the bands observed in western blots were forms of C/EBPβ. This finding suggested that the observed bands were either C/EBPβ isoforms or homo- and heterodimers formed between isoforms or other C/EBP molecules.

There are reports discussing the effect of PMA in intracellular signaling molecules. For example, it has been reported that PMA inhibits PKR activity in IFNα-treated mouse fibroblast cells (Zhou et al., 2005). Also, PMA and LPS together promote C/EBPβ mRNA translation in U937 cells (the effect of LPS or PMA alone was not studied) (Natsuka et al., 1992). However, PKR degradation or increased C/EBPβ expression was not observed

after PMA treatment (data not shown) but the expression of PKR and C/EBPβ were found to be dependent on amino acid composition of the expressed HLA-B27 HCs as after PMA treatment prior to LPS stimulation (0 hour time point) the expression of these molecules differs in B27g and H9F cells compared with mock and E45M cells.

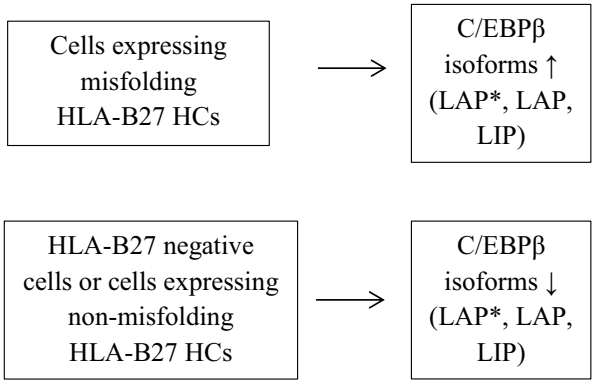

Figure 14. CCAAT enhancer binding protein beta (C/EBPβ) expression is increased in U937 cells expressing misfolding HLA-B27 heavy chains (HCs) compared with cells expressing non-misfolding HLA-B27 HCs.

5.3.1 CEBPβ expression was PKR-dependent

The role of PKR activity on C/EBPβ expression in U937 monocytic macrophages was studied with a specific PKR inhibitor (PKR+) (II: Figure 5A). The specificity of the inhibitor was confirmed by using a control compound for PKR inhibition (PKR-). The inhibitor and control compound share the similar structure and functionality, but the control is unable to inhibit PKR activity. After LPS stimulation in mock-transfected cells, inhibition of PKR activity decreased the expression of C/EBPβ to an undetectable level (II: Figure 5A). The phenomenon observed was dependent on LPS stimulation, since in unstimulated mock cells, no effect on C/EBPβ expression was observed upon PKR inhibition. In LPS-stimulated B27g cells, inhibition of PKR activation decreased C/EBPβ expression, but all isoforms and dimers were still highly expressed. Thus, in LPS-stimulated cells that did not express misfolding HLA-B27 HCs, active PKR regulated C/EBPβ expression. However, in HLA-B27-expressing cells, C/EBPβ expression was at least partially regulated by mechanisms that are unrelated to PKR.

5.3.2 PKR activity effected on cell death

It was studied whether the altered regulation of PKR in HLA-B27 expressing cells is linked to cell death. The inhibition of PKR activity lead to increased cell death both in HLA-B27-positive (B27g) and -negative (mock) cells, even without any stimulation (other than PMA, II: Figure 6). Interestingly, LPS stimulation did not have any effect on cell death in B27g cells whereas in mock cells, LPS induced cell death, indicating that B27g cells are more tolerant to LPS. However, PKR inhibition by siRNA had a

dramatic effect as most cells died. Therefore, PKR siRNA-treated cells could not be infected with *Salmonella*.

5.4 Increased HuR expression but decreased cleavage in HLA-B27 expressing cells (III)

The role of ELAVL1/HuR was studied in HLA-B27 expressing cells. The total expression of HuR was similar in all transfectants without stimulation or after LPS stimulation (III: Figure 1A). Moreover, neither LPS stimulation nor *Salmonella* infection influenced HuR expression in any of the transfectants (III: Figure 1B, C). However, amount of full length HuR (36 kDa) and two HuR CPs (CP1; 24 kDa and CP2; 8 kDa) varied (III: Figure 1B, C). Even without a stimulation (other than PMA), the amount of full length HuR was increased in cells expressing misfolding HLA-B27 HCs (B27g and B27.H9F) compared with nonmisfolding (B27.E45M) and control (mock) cells. Amount of CPs, instead, was decreased in misfolding HLA-B27 expressing cells (Figure 15.). After LPS stimulation or *Salmonella* infection, amount of full length HuR was increased in cells expressing misfolding HLA-B27 HCs (B27g and B27.H9F) compared with cells expressing properly folding HCs (B27.E45M) or HLA-B27 negative cells (mock) (III: Figure 1B, C). Furthermore, HuR cleavage was disturbed in HLA-B27 expressing cells as the amount of CPs was lower in these cells. Thus HuR expression pattern was linked to the amino acid composition of HLA-B27 B-pocket that in turn is linked to the misfolding feature of the HLA-B27 molecule.

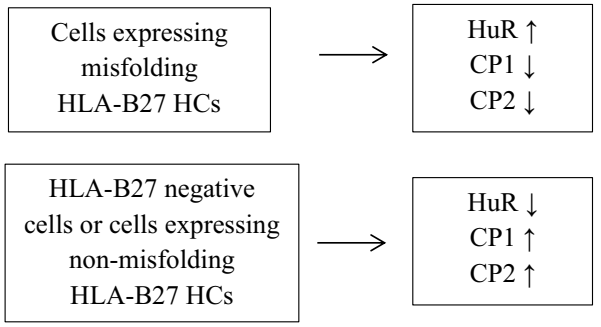

Figure 15. Despite increased human RNA binding protein (HuR) expression, generation of HuR cleavage products (CP1 and CP2) is disturbed in U937 cells expressing misfolding HLA-B27 heavy chains (HCs) compared with cells expressing non-misfolding HLA-B27 HCs.

5.4.1 HuR was remotely dependent on PKR in HLA-B27 expressing cells after infection with *Salmonella*

A specific PKR inhibitor (PKR+) was used to investigate the role of PKR in HuR regulation in HLA-B27 expressing human monocytic macrophages (III: Figure 2A, B). A control compound for PKR inhibition (PKR-, according to the manufacturer, apart from PKR inhibition, the control compound has the same influence on cell behavior than the actual inhibitor) was also used. PKR regulated HuR expression in both HLA-B27 positive and negative cells. Inhibition of PKR activity had only a minor effect on

full length HuR expression in B27g cells after LPS stimulation or *Salmonella* infection (III: Figure 2A, B). In PKR-inhibited mock, B27.E45M and B27.H9F transfectants, increased amount of full length HuR is observed after LPS stimulation whereas infection induced HuR expression only in non-misfolding mutant transfectant B27.E45M and control cells (mock) (III: Figure 2A). CP generation was induced in all transfectants treated with PKR inhibitor after LPS stimulation or bacterial infection.

5.4.2 HuR expression was not p38-dependent in HLA-B27 expressing cells

p38 activation was inhibited with a p38 inhibitor (SB202190). A control compound for p38 inhibition (SB202474) was also used (according to the manufacturer, apart from p38 inhibition, the control compound has the same influence on cell behavior than the actual inhibitor). In control cells (mock), inhibition of p38 activity after *S.* Enteritidis infection induced a decrease in the amount of full length HuR (III: Figure 3B). Similar decrease was also observed in non-misfolding B27.E45M cells. However, in cells expressing misfolding HLA-B27 HCs (B27g and B27.H9F), p38 inhibition had no effect. Similar, but less profound decrease is observed in mock and B27.E45M after LPS stimulation and consistent with the observations after infection, no effect on B27g or B27.H9F was detected (III: Figure 3A). Moreover, another p38 MAPK inhibitor, BIRB 796 that differs structurally from SB202190 but is a slow binder to its substrates, had no effect on HuR expression pattern in B27g or B27.H9F cells after LPS stimulation (III: Figure 4A). However, the effect of BIRB 796 on HuR expression in mock and B27.E45M was similar, but less profound than the effect observed after SB202190 treatment. Thus to ensure that the decrease in HuR expression observed in cells that do not contain misfolding HCs was caused by the inhibition of p38 activity rather than some other molecules, other inhibitors were used. Besides p38, SB202190 is known to effect on Src, Casein I and Raf1 kinase activities (Bain et al., 2007). The inhibitors targeted against these kinases had no notable effect on full length HuR or CP expression in any transfectant, despite the small but noteworthy decrease of full length HuR and CP1 in B27g cells after Raf1 inhibition (III: Figure 4B).

5.5 TNFα secretion was PKR-dependent and IL-10 secretion was PKR- and p38 MAPK-dependent (III)

It was studied whether p38- and/or PKR-dependent TNFα and IL-10 secretion is altered in HLA-B27 expressing U937 cells (III: Figure 5A-D). Only negligible TNFα and IL-10 secretion was detected without any stimulation (other than PMA). Mock, B27g and B27.H9F cells secreted similar amounts of both cytokines after LPS stimulation whereas in E45M cells cytokine secretion was undetectable. Inhibition of p38 activity had no effect on TNFα secretion in any of the transfectants (III: Figure 5B). In contrast, TNFα secretion was completely dependent on PKR activity in all transfectants since the inhibition of PKR activity downregulates TNFα secretion to an undetectable level (III: Figure 5A). Moreover, IL-10 secretion was both PKR- and p38-dependent in all transfectants (III: Figure 5C, D). Thus TNFα and IL-10 secretion were independent of the expression of misfolding HLA-B27 HCs.

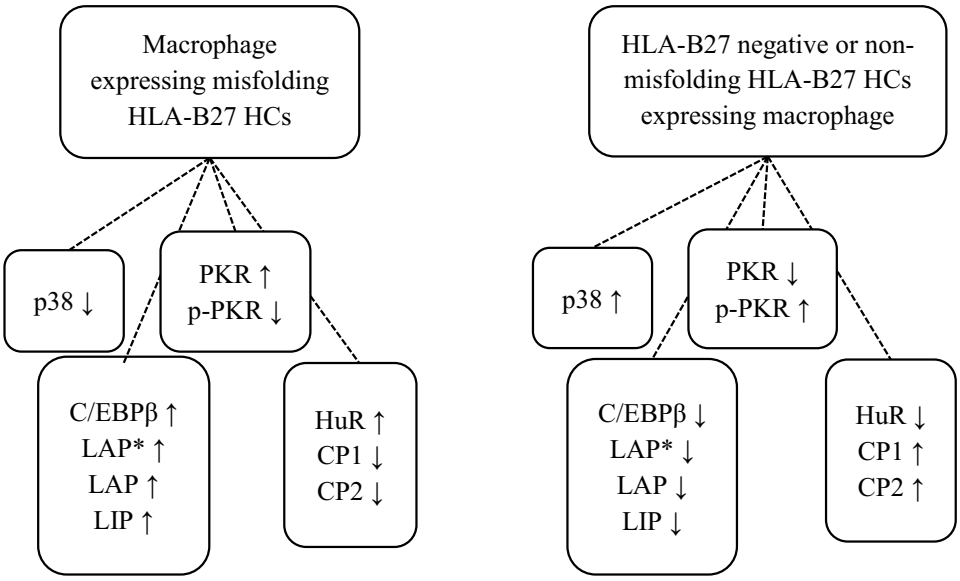

Figure 16. An overview of the main results. Activation, expression or function of intracellular signaling molecules in U937 monocytic macrophages expressing misfolding HLA-B27 heavy chains (HCs) or in non-misfolding (or HLA-B27 negative) cells compared with each other.

6 DISCUSSION

6.1 Modulation of intracellular signaling by HLA-B27

Despite extensive studies, the mechanisms behind the pathogenesis of ReA and other SpAs are unclear. HLA-B27 certainly has an important role but how it contributes to the disease pathogenesis is still unknown. However, several theories concerning HLA-B27 antigen presenting and non-antigen presenting functions exist. There is evidence suggesting that the host-pathogen interaction between ReA-triggering bacteria and HLA-B27 expressing host cell is abnormal. This idea is supported by the known fact that ReA patients have prolonged antibody response towards the triggering pathogen supporting the idea of persisting pathogen or pathogenic components in the host (Granfors et al., 1990, Granfors, 1992, Mäki-Ikola et al., 1991). Also, it is known that macrophages are important in preventing the occurrence of a chronic infection. Bacteria, e.g. *Salmonellae,* try to exploit host cell, often a macrophage, by using it to enable proliferation and avoid elimination by the immune system. The expression of HLA-B27 in human monocytic macrophages modulates the intracellular environment. Bacterial elimination is disturbed and the pathogen is able to survive (Laitio et al., 1997). The phenomenon is dependent on the amino acid composition of the peptide binding groove in the B pocket of HLA-B27 HCs (Penttinen et al., 2004). The results suggest that the activation of the p38 MAPK pathway is one of the factors controlling *Salmonella* replication in human monocytic U937 cells and human peripheral blood cells (I). Interestingly, this dependence is disrupted in HLA-B27 expressing cells and can also be linked to the composition of the peptide binding groove responsible for the misfolding and homodimerizing features of HLA-B27 HCs. Moreover, *Salmonella* effectors may inactivate p38 by altering the p38 phosphorylation loop (Zhu et al., 2007). The inhibition of p38α is sufficient for an anti-inflammatory response in an *in vivo* mouse model (O'Keefe et al., 2007). Thus it can be speculated whether the p38α is downregulated in HLA-B27 expressing cells where p38 inhibition has almost no effect. If that is the case, it can be further speculated that normal immune response is not initiated when the cells are infected with *Salmonella* and consequently, the bacteria are not eliminated. This is supported by the fact that in HLA-B27 negative cells p38 inhibition disturbs intracellular bacterial replication. However, it is likely that the defect in the p38 signaling pathway takes place downstream of p38 rather than in the p38 itself since similar p38 activation was observed in HLA-B27 positive and negative U937 cells (I). Moreover, Th1 response is found to be p38-dependent in a mouse model after *Leishmania* infection (Yang et al., 2010). As Th1 response is crucial for the resistance of intracellular bacterial survival in macrophages, a defect in p38 activation might offer an explanation for intracellular bacterial survival.

Mouse homologue of C/EBPβ, NF-IL6, has a central role in regulating the survival of *Salmonella* in mouse macrophages as C/EBPβ knockout mice are more susceptible to bacterial infection due to impaired bacterial elimination (Tanaka et al., 1995). C/EBPβ has several isoforms which are regulated through transcriptional and

posttranscriptional mechanisms. The smallest isoform, LIP, regulates intracellular viral replication (Komuro et al., 2008). In HLA-B27 expressing cells C/EBPβ is overexpressed and mainly PKR-independent upon LPS-stimulation (II). Since inactive PKR regulates its own expression, it is possible that HLA-B27-expressing cells produce high amounts of PKR that cannot be activated by phosphorylation and thus fails to regulate the expression of C/EBPβ. Although C/EBPβ expression in HLA-B27 cells is not PKR-dependent like in control cells, it was strongly expressed in these cells upon LPS stimulation (II). This suggests that other regulatory mechanism(s) have compensated PKR and are involved to maintain the C/EBPβ expression. HuR can be linked to the prolonged C/EBPβ expression as it is able to bind to the UTR and stabilize mRNA through it (Johann et al., 2008, Licata et al., 2009, Suswam et al., 2005, Zhang et al., 2008). Thus HuR extends the time frame for protein expression by enhancing translation (Bergalet et al., 2011). However, it has been reported that HuR binding may decrease C/EBPβ expression as it results in the nuclear retention of C/EBPβ mRNA (Cherry et al., 2008). As HuR expression is increased and its cleavage is dysregulated in HLA-B27 cells it can be speculated whether also HuR binding to mRNA targets is disturbed. Thus there are two possible explanations for increased C/EBPβ expression in HLA-B27 expressing cells. Increased full length HuR may stabilize and enhance its translation, or impaired HuR binding may lead to the accumulation of cytoplasmic mRNA leading to increased protein production.

It is still unclear how the expression of HLA-B27 HCs modulates the expression, activation and functionality of the intracellular signaling molecules. One possible mechanism is the regulation of autophagy. In addition to ERAD, proteins and even pathogens may be degraded by this catabolic process that works through the lysosomal machinery. For example, misfolding proteins that accumulate in the ER can be degraded by autophagy (Fujita et al., 2007). Furthermore, impaired UPR can lead to the induction of autophagy (Matus et al., 2009). Interestingly, despite the presence of misfolded HLA-B27 HCs, UPR has not been detected in the stably transfected U937 cells (Penttinen et al., 2004). Moreover, the findings show that STAT1 is hyperphosphorylated in cells expressing misfolding HLA-B27 (Ruuska et al., 2012) and PKR is hypophosphorylated. STAT1 functions as a PKR inhibitor (Wong et al., 2001) and PKR has been linked to the activation of autophagy in macrophages (Sir and Ou, 2010). Furthermore, it has been suggested that PKR has an important role in the activation of mechanisms that protect against non-viral pathogens for PKR is a prerequisite mediator of CD40-TRAF-autophagy pathway (Ogolla et al., 2013). Thus it can be suggested that this regulatory loop may lead to a situation where PKR is only modestly active in stably transfected HLA-B27-expressing cells, autophagy is not induced and bacteria may persit in the cells.

As mentioned above, UPR was not detected in stably transfected cell line model used in this study (Penttinen et al., 2004). Induction of BiP or CHOP was not observed despite the presence of misfolding HCs. Thus one possible mechanism for increased C/EBPβ expression detected upon stimulation in HLA-B27 positive U937 cells is disturbed UPR activation despite the continuous synthesis of misfolded proteins. CHOP functions as a dominant negative inhibitor of C/EBPs (Oyadomari and Mori, 2004). In addition, CHOP is activated by the p38 signaling pathway (Maytin et al.,

2001) that is dysregulated in HLA-B27 expressing cells. Thus in the absence of CHOP induction, C/EBPβ expression may increase. C/EBPβ regulates various factors involved in the inflammatory response and altered C/EBPβ expression may be an important factor behind the altered intracellular milieu that favors ReA-triggering bacteria in HLA-B27 expressing cells. It is also possible that misfolded proteins are degraded through ERAD and thus do not accumulate and trigger UPR. However, highly efficient ERAD should lead to a loss of the functional protein (Reviewed in Colbert et al., 2014) and in this study, HLA-B27 molecules are found on the surface of HLA-B27-transfected U937 cells.

6.2 Modulation of cell survival by HLA-B27

Salmonella virulence is controlled by genes encoded in SPIs. SPI2 mainly regulates intracellular bacterial survival and replication (Reviewed in Fàbrega and Vila, 2013) and *Salmonella* avoids elimination by the host cell by regulating *Salmonella*-induced cell death. SPI2 is required for *Salmonella*-induced PKR-mediated macrophage apoptosis (Hsu et al., 2004). PKR is overexpressed in human monocytic macrophages expressing misfolding HLA-B27 HCs (II). In these cells, PKR is also hypophosphorylated. Thus the cells express increased amounts of PKR, but the PKR activation by Thr451 phosphorylation (indispensable for the PKR kinase activity (Romano et al., 1998) is disrupted. PKR activation is inhibited during acute human immunodeficiency virus (HIV) replication (Clerzius et al., 2009). It has been suggested that HIV only replicates in cells, where PKR activity is repressed. It can be speculated that decreased PKR activity in HLA-B27 expressing cells might enable intracellular *Salmonella* replication similarly to HIV replication. Intriguingly, HLA-B27 is linked to mitigation of viral infections e.g. HIV and hepatitis C (Reviewed in Neumann-Haefelin, 2013). However, this feature of HLA-B27 is linked to the antigen presentation to T cells (Reviewed in Neumann-Haefelin, 2013) and thus does not contradict the possible intracellular effects on pathogen replication in macrophages. In normal conditions, PKR is localized in the cytoplasm in an unphosphorylated form and nuclear accumulation of a phosphorylated form is triggered by stress signals (Reviewed in Dabo and Meurs, 2012). It has also been suggested that nuclear accumulation of p-451 PKR sensitizes cells to PKR inhibition which leads to apoptosis (Blalock et al., 2011). Interestingly, when PKR was blocked with siRNA transfection in control (mock) cells, the cells died and expereiments could not be performed (II). Thus it would be of interest to study the cellular localization of PKR.

PKR is involved in p38-mediated apoptosis in mouse macrophages (Hsu et al., 2004). The misfolding of newly synthetized molecules in the ER is linked to the activation of PKR (Zhang et al., 2006). Hence, it is possible that PKR fails to activate p38-mediated apoptosis in HLA-B27-expressing cells. This idea is indirectly supported by the finding that LPS-stimulated HLA-B27-expressing U937 cells show increased tolerance to cell death (II). Along PKR, the transcription factor C/EBPβ is known to be involved in the regulation of apoptosis. Consistent with findings by others (Natsuka et al., 1992), increased C/EBPβ expression induced by LPS stimulation and *Salmonella* infection

was observed in HLA-B27-expressing cells (II). In non-HLA-B27 expressing cells, induced C/EBPβ expression is dependent on PKR activity. However, in HLA-B27 expressing cells, C/EBPβ expression seems to be only modestly PKR-regulated. Thus HLA-B27 expression indirectly leads to increased C/EBPβ expression upon stimulation by bacteria or bacterial compartments. C/EBPβ expression in non-stimulated as well as in HLA-B27 expressing macrophages is regulated by some other factors than PKR, for example p38-dependent phosphorylation (Horie et al., 2007) whereas PKR regulates C/EBPβ expression after LPS stimulation or infection in non-HLA-B27 expressing cells. Taken together, the PKR involved signaling is complex. Whether PKR is involved in a certain signaling event and how it effects, might be dependent on the cell type and stress stimulus (Reviewed in Dabo and Meurs, 2012). Also, PKR can be regulated in a time-dependent manner. For example HI virus induces PKR activation by phosphorylation. However, PKR activation is inhibited during acute HIV replication (Clerzius et al., 2009). In addition, dsRNA induces local joint inflammation through NF- κB signaling in mice (Zare et al., 2004) and in articular chondrocytes, TNFα stimulation upregulates PKR and eIF2α phosphorylation leading to arthritogenic cartilage degradation (Gilbert et al., 2002). In an animal model, PKR is activated in early osteoarthritis and has a role in arthritic disease onset and progression (Gilbert et al., 2004). Thus PKR signaling is important in the pathogenesis of various diseases through complex mechanisms.

In cellular stress, two HuR cleavage products CP1 and CP2 are generated. CPs are linked to the promotion of apoptosis (von Roretz and Gallouzi, 2010). The expression of HuR and the generation of the two CPs are altered in U937 monocytic macrophages that express HLA-B27 HCs (III). In HLA-B27 negative cells, the amount of CPs generated is higher than in cells expressing misfolding HLA-B27 HCs. This phenomenon is dependent on the amino acid composition of the HLA-B27 HCs as altered HuR expression and cleavage is dependent on the glutamic acid 45 in the B pocket that is at least partially responsible for the misfolding feature of the HLA-B27 molecule. HuR cleavage is regulated by unphosphorylated PKR that triggers cleavage by activating the caspase-8/caspase-3 pathway (von Roretz and Gallouzi, 2010). Moreover, HuR cleavage after *Salmonella* infection in the HLA-B27 expressing cells is less dependent on PKR activity than without infection or in infected HLA-B27 negative cells. Impaired HuR cleavage delays cell death (Mazroui et al., 2008, von Roretz and Gallouzi, 2010) and HLA-B27-expressing monocytic macrophages are more tolerant to cell death after LPS stimulation (II). However, PKR is overexpressed but hypophosphorylated in HLA-B27 expressing cells, higher HuR cleavage rate was expected (III). As the cleavage rate detected was in fact lower, it can be suggested that not only signaling dependent on the phosphorylation of PKR is disturbed in these cells, but also unphosphporylated PKR linked to HuR cleavage (von Roretz and Gallouzi, 2010), might be dysfunctional. In addition, PKR regulated *Salmonella*-induced cell death is p38-dependent (Hsu et al., 2004) and also p38 signaling is disturbed in HLA-B27 expressing cells. It has been reported that TNFα delays apoptosis in human eosinophils (Kankaanranta et al., 2014). TNFα is important in the pathogenesis of SpA as the disease activity is decreased in response to TNFα blockers (Sieper, 2011). Thus

it is possible that in HLA-B27 expressing cells, *Salmonella* may reside in the host cell, for example in SCVs, in order to avoid elimination and enable bacterial persistence.

6.3 Regulation of cytokines in HLA-B27 expressing cells

p38 and its downstream target MK2 are known to be involved in the post-transcriptional regulation of TNFα (Kotlyarov et al., 1999), for p38 and MK2 activation by phosphorylation leads to the activation of TNFα translation. Moreover, the inhibition of p38 leads to induced IL-12 but decreased TNFα expression (Yang et al., 2010). The results show that p38-related signaling is dysregulated in HLA-B27 positive cells (I). In addition, the cells secrete increased amount of TNFα compared with HLA-A2 transfectants (Penttinen et al., 2002). However, after LPS stimulation, TNFα secretion is similar in HLA-B27 transfectants compared with mock-transfected control cells indicating that the expression of HLA-B27 does not increase the TNFα secretion but it can rather be speculated that the expression of HLA-A2 might downregulate it (III). The secretion of IL-10, a potent anti-inflammatory cytokine was found to be increased in HLA-B27 expressing U937 transfectants upon LPS stimulation and *Salmonella*-infection compared with HLA-A2 transfectants (Ekman et al., 2002). IL-10 is known to suppress p38 activation in U937 cells (Krishnamurthy et al., 2009, Rajasingh et al., 2006). Similarly to TNFα secretion, LPS-triggered IL-10 secretion is not influenced by the expression of HLA-B27 compared with mock-transfected cells (III). It can be suggested that the expression of HLA-A2 modulates the secretion of TNFα and IL-10 and these modulatory effects are disrupted in HLA-A2 negative HLA-B27 and mock cells possibly due to misfolding or absence of the functional HLA molecules.

HuR overexpression substantially increases the half-life of many short-lived mRNAs like TNFα that are stabilized through multiple ARE units (Dean et al., 2001). p38 activation also leads to a rapid cytoplasmic accumulation of HuR (Farooq et al., 2009), a phenomenon that is a known stabilizer of TNFα mRNA (Rajasingh et al., 2006). Moreover, HuR expression and cytoplasmic accumulation together with the activation of p38/MK2 pathway regulate the translation of TNFα and other ARE-containing mRNAs (Tiedje et al., 2012). IL-10 inhibits HuR in U937 cells (Krishnamurthy et al., 2009, Rajasingh et al., 2006). Full length HuR (36 kDa) expression is increased in cells expressing misfolding forms of HLA-B27 where intracellular bacteria are able to replicate and the p38 pathway is dysregulated (III). HuR is not downregulated although IL-10 is synthetized. Thus anti-inflammatory effects such as downregulation of TNFα secretion normally mediated by IL-10 are not conducted. In line with other studies (Cabanski et al., 2008), it was observed that PKR regulates TNFα secretion in U937 cells. Moreover, PKR is a prerequisite for TNFα and IL-10 secretion. p38 inhibition downregulates HuR expression in mock but does not effect on TNFα secretion in either mock or HLA-B27 transfectants. Thus, it is unlikely that misfolding of HLA-B27 HCs or altered HuR regulation have major impact on TNFα secretion in U937 monocytic macrophages. Contrary to TNFα secretion, IL-10 secretion is regulated also by p38. However, IL-10 secretion is similarly controlled by PKR and p38 both in HLA-B27-

expressing and control cells being independent of the expression of HLA-B27. Thus it is not likely that defect in IL-10 regulation would explain altered HuR expression.

6.4 HLA-B27, intracellular signaling and pathogenesis of ReA

Intracellular signaling molecules are of importance in the pathogenesis of various diseases. For example, PKR is linked to Alzheimer's disease by its apoptosis mediating function, intestinal inflammation through mitochondrial stress and UPR, and arthritis through pro-inflammatory cytokine production and cartilage degradation (Gilbert et al., 2012, Gilbert et al., 2002, Gilbert et al., 2004, Page et al., 2006, Rath et al., 2012). However, the role of signaling molecules in the pathogeneses of SpAs is complex as it has been stated that dsRNA-induced arthritis is not mediated by PKR in a mouse model (Magnusson et al., 2006). As the pathogeneses of SpAs differ from each other, also the mechanism how HLA-B27 predisposes to the disease may differ. To conclude, intracellular signaling related to p38, PKR and HuR is altered in HLA-B27 expressing monocytic macrophages (Figure 12.). Thus it is also possible that these findings offer a partial explanation how the expression of HLA-B27 alters the intracellular environment in favor of the ReA triggering bacteria. The exact mechanism how HLA-B27 HCs modulate the intracellular signaling remains to be solved although the amino acid composition and especially glutamic acid at the position 45 at the peptide binding groove seem to be of importance. Taken the results together, it can also be suggested that there is a correlation between HLA-B27 amino acid composition, the expression of misfolding HLA-B27 molecules and the expression of the intracellular signaling molecules. It was shown that the expression and / or activation of several molecules known to involve inflammatory response or *Salmonella* elimination in macrophages is altered in cells expressing misfolding HLA-B27 HCs. The effect of HLA-B27 on cellular environment is dependent on E45 residue in the peptide binding groove that is at least partially responsible of the misfolding feature of the HLA-B27 molecule. To conclude, the expression of HLA-B27 modulates the inflammatory response induced by ReA triggering bacteria by affecting intracellular signaling molecules. The results suggest that the expression of HLA-B27 alone is sufficient to alter the intracellular environment. Since p38, PKR, C/EBPβ and HuR have a central role in the regulation of the inflammatory response; the findings provide evidence that HLA-B27 modulates the interaction between host cell and ReA triggering bacteria. However, p38, PKR, C/EBPβ and HuR are not likely to be the only molecules affected by HLA-B27 expression. Thus an extensive understanding of the role of HLA-B27 and its misfolding HCs in cellular signaling remains to be achieved.

6.5 Methodological considerations

6.5.1 U937 cell line and HLA-B27 transfectants

To study whether the expression of HLA-B27 HCs in monocytic cells modifies the intracellular environment, U937 monocytic cells were stably transfected. The genomic

clone of HLA–B*2705 DNA, an HLA-B27 subtype that is linked to the misfolding of HLA-B27 HCs and is associated to SpAs was used. HLA-B*2705 subtype is common and it is found in all populations. Moreover, it has been considered as the original HLA-B27 molecule from which the other subtypes have evolved (Reviewed in Reveille, 2006). Mock-transfected cells treated similarly than HLA-B27 transfected cells were used as controls. In addition, cells expressing mutated forms of HLA-B*2705 HCs were used. With these mutated transfectants, it was possible to study whether the amino acid composition and thus the misfolding and/or homodimerizing features of HLA-B27 HCs play a role in the formation of intracellular milieu. Furthermore, the interaction of *Salmonella* and host cell, or host response after stimulation with bacterial compartments was studied. Although it must be accepted that results obtained from experiments with transfected cell lines might not totally reflect the situation in patients, the use of transfected cells was regarded reasonable. Immortalized cell lines are widely used to study host-bacteria interactions and transfected cell lines provide a convenient tool for obtaining important information about human cells. As the study was conducted with transfected cell lines it was possible to eliminate the variance caused by genetic differences, and it was possible to perform the experiments as controlled as possible in order to get reliable and repeatable results. Thus transfectants were found to be more convenient than primary cells. Comparison between HLA-B27 expressing cells and controls was possible as the cells were treated similarly in the same conditions and the sole difference was the expression of HLA- B*2705. New batches of transfected cells were introduced every three months and HLA-B27 expression was ensured by flow cytometry analysis. Statistical significance was not always achieved as the experiments must be repeated at least five times in order to be able to do reliable statistical analysis. However, at least three independent repetitions were conducted in all experiments.

In addition to transfected cell lines, transgenic animal models have been generated to study the role of HLA-B27 expression in the pathogenesis of SpAs. It must be noted that results obtained from experiments with human cell lines and cells from transgenic animals may yield different results. For example, *S.* Typhimurium causes gastroenteritis in human and typhoid-like systemic disease in mice. Also the regulation of NO synthase, a factor important in microbial resistance, differs significantly in murine and human macrophages (de Vera et al., 1996). HLA-B27 upregulation leads to ER stress and UPR in rat macrophages (Turner et al., 2005). However, in HLA- B27-expressing U937 cells UPR has not been detected (Penttinen et al., 2004). It can be speculated that the response to an acute increase in the expression of a misfolding protein can differ from the response observed in cells where misfolding molecules are constantly expressed. A natural consequence of the unresolved ER stress is apoptosis. In order to avoid that, it is possible that transfected cells expressing high levels of a potentially toxic protein survive because of the altered regulation of the pro- and antiapoptotic pathways. If that is the case, the results obtained in this study with stable HLA-B27 transfected monocytic macrophages might differ from freshly isolated peripheral blood mononuclear cells. In addition, UPR occurring during monocyte to macrophage differentiation protects from later UPR triggering signals (Dickhout et al., 2011) and monocytic cells used in all experiments were maturated with PMA to

macrophages. Also, intracellular signaling in an immortalized cell line can be different from what is observed in primary cells. For example, despite several attempts, IFNγ or IL-12 secretion from U937 cells has not been detected. Nonetheless, these results strongly suggest that HLA-B27 expression by itself is enough to dramatically modify the intracellular environment.

6.5.2 Inhibitors used in this study

Commercially available inhibitors targeted against different intracellular molecules were used to study the relevance of these molecules in HLA-B27 expressing cells. However, the inhibitors often affect other molecules than the specified target molecule, leaving it difficult to speculate what is the molecule behind the possible effect observed after the cells are treated with the inhibitor. Whenever possible, a negative control sharing the similar chemical structure than the inhibitor was used to ensure the specificity. Moreover, another, structurally different p38 inhibitor than SB202190 used in most experiments was used to study the role of p38 activity on HuR expression. When BIRB 796 was introduced to the cells according to the similar protocol used in other experiments with inhibitors, no effect on HuR expression was observed. As BIRB 796 is known to be a slow binder, the incubation time was increased and similar but less profound effect on HuR was observed than after incubation with SB202190. To further ensure that the effect observed on HuR expression and CP generation was induced by the inhibition of p38 catalytic activity, it was tested whether some of the other kinases influenced by SB202190 regulate HuR. SB202190 also inhibits casein kinase 1 delta (CK1δ), receptor-interacting serine-threonine kinase 2 (RIPK2) and cyclin G associated kinase (GAK), 21 %, 7 % and 0 % of the activity remains after treatment, respectively (Bain et al., 2007). After other inhibitors targeting these molecules were used, no significant effect on HuR expression was observed. To conclude, there might be several kinases involved in HuR regulation that are also affected by treatment with SB202190 but the findings strongly suggest that p38 is among the HuR regulators in U937 monocytic macrophages.

7 SUMMARY AND CONCLUDING REMARKS

The mechanisms behind the pathogenesis of ReA how HLA-B27 contributes to the disease susceptibility and outcome are still unclear. It is known that the compartments of disease triggering bacteria are able to persist in the ReA patient. There are several theories trying to explain the exact role of HLA-B27 in development and outcome of ReA. The misfolding and homodimerization features of HLA-B27 HCs are all likely to play a role and partially explain the relevance of HLA-B27. It can be concluded that the intracellular environment of the HLA-B27 expressing cells is altered and the host-pathogen interaction between the triggering bacteria and host cells is disturbed.

Macrophages are important in preventing the occurrence of a chronic infection. As for the bacteria, the intracellular proliferation and avoiding the elimination by macrophages are crucial. It has been suggested that the expression of HLA-B27 in human monocytic macrophages modulates the intracellular environment enabling the survival of the pathogen. In addition, the amino acid composition of the peptide binding groove of HLA-B27 HCs is linked to the bacterial survival. This study was undertaken to further elucidate the mechanisms behind the prolonged bacterial persistence. It was shown that the expression and / or activation of several molecules known to involve inflammatory response or *Salmonella* elimination in macrophages is altered in cells expressing misfolding HLA-B27 HCs. The results suggest that the activation of the p38 MAPK pathway is one of the factors controlling *Salmonella* replication in human monocytic U937 cells and human peripheral blood cells. Altered p38 signaling is dependent on the glutamic acid at the position 45 at the peptide binding groove. Interestingly, this amino acid is linked to the misfolding feature of HLA-B27 HCs. PKR is overexpressed but hypophosphorylated and C/EBPβ is overexpressed and mainly PKR-independent upon LPS-stimulation in HLA-B27 expressing cells. Moreover, PKR involves mechanisms targeted against external pathogens and C/EBPβ has a central role in regulating the survival of *Salmonella* in mouse macrophages. Also the expression of HuR and the generation of the two CPs are altered in cells expressing HLA-B27 HCs. In HLA-B27 positive cells, the amount of CPs generated is low although the full length HuR is highly expressed. Interestingly, the altered expression and activation of signaling molecules is also dependent on the HLA-B27 amino acid composition linked to the misfolding.

The findings of this study strongly suggest that the expression of HLA-B*2705 subtype alone is sufficient to modulate the intracellular environment of human monocytic macrophages by altering the expression and/or activation of intracellular signaling molecules and leading to an abnormal response to cellular stress. Understanding of the intracellular pathways modified by genetic features and targeted by bacteria in order to facilitate replication and survival may lead to better understanding of disease pathogenesis and thus provide novel approaches in treatment.

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