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MODULATION OF SIGNALING MOLECULES BY HUMAN LEUCOCYTE ANTIGEN B27; SPECIAL REFERENCE TO STAT1

Marja Ruuska

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University of Turku

Faculty of Medicine

Department of Biomedicine

Medical Microbiology and Immunology

Turku Doctoral Programme of Molecular Medicine

and

Department of Infectious Diseases Surveillance and Control, National Institute for Health and Welfare

Supervised by

Docent Kaisa Granfors, PhD
Department of Infectious Diseases Surveillance and Control
National Institute for Health and Welfare
Turku, Finland

Markus Penttinen, MD, PhD
Medical microbiology and Immunology
Department of Biomedicine
University of Turku, Turku Finland

Reviewed by

Docent Sampsa Matikainen, PhD
Unit of Systems Toxicology
Finnish Institute of Occupational Health, Finland

Professor Outi Vaarala, MD, PhD
Institute of Clinical Medicine
University of Helsinki, Helsinki, Finland

Opponent

Professor Olli Vainio, MD, PhD
Institute of Diagnostics
Department of Medical Microbiology and Immunology
University of Oulu
Oulu, Finland

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Learn from yesterday, live for today, hope for tomorrow.
The important thing is not to stop questioning.

Albert Einstein

ABSTRACT

Marja Ruuska

Modulation of signaling molecules by human leucocyte antigen B27; special reference to STAT1

The Department of Biomedicine, Medical Microbiology and Immunology, University of Turku, Finland and the Department of Infectious Diseases Surveillance and Control, National Institute for Health and Welfare, Turku, Finland.
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Reactive arthritis (ReA) is an inflammatory joint disease, which belongs to the group of Spondyloarthritis (SpA). It may occur after infections with certain gram-negative bacteria such as *Salmonella* and *Yersinia*. SpAs are strongly associated with the human leucocyte antigen (HLA)-B27. Despite active research, the mechanism by which HLA-B27 causes disease susceptibility is still unknown. However, HLA-B27 has a tendency to misfold during assembly. It is possible that the misfolding of HLA-B27 could alter signaling pathways and/or molecules involved in inflammatory response in cells. We have earlier discovered that in HLA-B27-positive cells the interaction between the host and causative bacteria is disturbed. Our recent studies indicate that the expression of HLA-B27 may alter certain signaling molecules by disturbing their activation.

The aim of this study was to investigate whether the expression of HLA-B27 disturbs the signaling molecules, especially the phosphorylation of transcription factor STAT1. STAT1 is an important mediator of inflammatory responses. Our results show that the phosphorylation of the STAT1 is significantly altered in HLA-B27-expressing U937 monocytic cells compared with control cells. STAT1 tyrosine 701 is more strongly phosphorylated in HLA-B27-expressing cells; whereas the phosphorylation of STAT1 serine 727 is prolonged. Phosphorylation of STAT1 was discovered to be dependent on protein kinase PKR. Furthermore, we found out that the expression of posttranscriptional gene regulator HuR was altered in HLA-B27-expressing cells. We also detected that HLA-B27-positive cells secrete more interleukin 6, which is an important mediator of inflammation. These results help to understand how HLA-B27 may confer susceptibility to SpAs.

Key words: spondyloarthritis, HLA-B27, misfolding, cell signaling, transcription factor STAT1, phosphorylation

TIIVISTELMÄ

Marja Ruuska

Ihmisen leukosyyttiantigeeni B27:n aiheuttamat muutokset signalointimolekyyleissä, erityisesti STAT1-proteiinissa.

Biolääketieteen laitos, Lääketieteellinen mikrobiologia ja immunologia, Turun yliopisto, Turku ja Tartuntatautiseurannan ja -torjunnan osasto, Terveyden ja hyvinvoinnin laitos, Turku.
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Reaktiivinen artriitti (ReA) on spondyloartriittien (SpA) ryhmään kuuluva niveltulehdussairaus. Se voi ilmetä tiettyjen bakteeritautien, kuten salmonellan ja yersinian aiheuttamien tulehdusten jälkitautina. Alttius sairastua ReA:iin ja muihin SpA:hin liittyy voimakkaasti yksilölliseen HLA-B27-kudostekijään. Aktiivisesta tutkimustyöstä huolimatta ei tiedetä millä mekanismilla HLA-B27 vaikuttaa sairastumisherkkyyteen. Tiedetään kuitenkin, että HLA-B27-molekyylillä on taipumus laskostua valmistumisvaiheessa epätäydellisesti. Tämä epätäydellinen laskostuminen saattaakin muuttaa sellaisia solunsisäisiä signalointireittejä ja/tai niihin osallistuvia molekyylejä, jotka vaikuttavat solun immuuni- ja tulehdusvasteisiin. Olemme aiemmin havainneet HLA-B27:n muuttavan isäntäelimistön ja bakteerin välistä vuorovaikutusta niin, että solujen kyky tappaa ja vastustaa *Salmonella enteritidiksen* lisääntymistä solujen sisällä on heikentynyt merkittävästi. Viimeaikaiset tulokset puolestaan osoittavat, että HLA-B27 vaikuttaa tiettyjen signalointiin osallistuvien molekyylien, kuten proteiinikinaasien p38 MAPK:n ja PKR:n, käyttäytymiseen poikkeavasti.

Tämän tutkimuksen tarkoituksena oli selvittää kuinka HLA-B27 vaikuttaa tulehdusreaktiossa tärkeisiin solunsisäisiin signalointimolekyyleihin. Erityisesti tutkittiin tulehduksen kannalta merkittävän signalointimolekyylin, transkriptiotekijä STAT1:n aktivoitumista fosforyloitumalla. Tulokset osoittavat että soluissa, jotka ilmentävät HLA-B27-kudostekijää, STAT1:n aktivaatio on muuttunut merkittävästi kontrollisoluihin verrattuna. STAT1:n tyrosiini 701 -tähde on fosforyloitunut voimakkaammin HLA-B27-positiivisissa soluissa kuin kontrollisoluissa. STAT1:n seriini 727 -tähteen fosforylaatio on puolestaan pitkittynyt HLA-B27-positiivisissa soluissa. Lisäksi, STAT1:n fosforylaation havaittiin olevan riippuvaista infektioiden aikana aktivoituvasta PKR:stä. STAT1:den poikkeuksellisen aktivaation lisäksi huomasimme, että lähetti RNA:ta stabiloivan proteiinin, HuR:n, ilmeneminen on poikkeavaa HLA-B27-positiivisissa soluissa. Havaitsimme myös, että HLA-B27-positiiviset solut eritettävät huomattavasti enemmän tulehduksessa välittäjäaineena toimivaa interleukiini 6:tta kuin kontrollisolut. Saadut tulokset auttavat ymmärtämään niitä mekanismeja, joilla HLA-B27 vaikuttaa sairauden syntyyn. Kun taudin syntymekanismit ymmärretään paremmin, on mahdollista kehittää tehokkaampia lääkkeitä niveltulehdusten hoitoon.

Avainsanat: spondyloartriitti, HLA-B27, proteiinien laskostuminen, solujen signalointi, transkriptiotekijä STAT1, fosforylaatio

TABLE OF CONTENTS

ABBREVIATIONS

ARE	AU-rich element
AS	Ankylosing spondylitis
ASAS	Assesment of SpondyloArthritis International Society
BiP	Binding immunoglobulin protein
C/EBP	CCAAT-enhancer-binding protein
cDNA	Complementary DNA
CIA	Collagen-induced arthritis
dsRNA	Double-stranded RNA
EA	Entero-associated arthritis
eIF	eukaryotic initiation factor
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERAD	Endoplasmic-reticulum-associated protein degradation
HBSS	Hank´s Balanced salt sodium
HC	Heavy chains
HLA	Human leukocyte antigen
HuR	Human antigen R
IBD	Inflammatory bowel disease
IFN	Interferon
IFNR	IFN receptor
IL	Interleukin
ISG	Interferon stimulated gene
JAK	Janus kinase
JNK	c-Jun N-terminal kinases
kbp	kilobase pair
KD	Kilo Dalton
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MAPK	Mitogen activated protein kinase
MHC	Major Histocompatibility complex
MKP-1	MAP kinase phosphatase 1
mRNA	messenger RNA
NF-κB	Nuclear factor kappa B
PIAS	Protein inhibitor of activated STAT
PKR	Double-stranded RNA-dependent protein kinase

PMA	Phorbol myristate acetate
PsA	Psoriatic arthritis
RA	Rheumatoid arthritis
ReA	Reactive arthritis
RPMI	Roswell Park Memorial Institute
SH2	Src Homology 2
SOCS	Suppressor of cytokine signaling
SpA	Spondyloarthritis
STAT	Signal transducer and activator of transcription
SUMO	Small Ubiquitin-like Modifier
TAP	Transporter associated with antigen processing
TC-PTP	T-cell protein tyrosine phosphatase
Th	T helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor
U937	human monocytic cell line
UPR	Unfolded protein response
uSpA	undifferentiated spondyloarthritis
UV	Ultra violet
XBP-1	X-box binding protein 1
β2m	β2-microglobulin

LIST OF ORIGINAL PUBLICATIONS

This study was based on the following four publications, which will be referred to in the text by Roman numerals (I-IV)

I Ruuska M, Sahlberg AS, Colbert RA, Granfors K, Penttinen MA. Enhanced phosphorylation of STAT1 is dependent on PKR signaling in HLA-B27 expressing U937 monocytic cells. *Arthritis and Rheumatism* 2012 Mar; 64(3):772-7.

II Ruuska M, Sahlberg AS, Granfors K, Penttinen MA. Phosphorylation of STAT-1 serine727 is prolonged in HLA-B27-expressing human monocytic cells. *PLoS One* 2013;8(1):e50684.

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

IV Ruuska M, Sahlberg AS, Granfors K, Penttinen MA. IL-6 secretion is elevated in HLA-B27-expressing U937 human monocytic cells. Manuscript

1. INTRODUCTION

Spondyloarthritides (SpA) are a group of chronic, inflammatory rheumatic diseases, which share several common clinical, genetic and pathophysiological features. SpAs can be divided into subgroups, one of which is an inflammatory joint disease, reactive arthritis (ReA). ReA is usually triggered by infection in gastrointestinal or urogenital tract caused by certain gram-negative bacteria such as *Salmonella, Yersinia* or *Chlamydia*. Diseases belonging to the group of SpAs are roughly estimated to affect around 1 % of world's population.

ReA, as all SpAs, has a strong association with human leucocyte antigen (HLA)-B27. Almost 80 % of the patients suffering from ReA are HLA-B27-positive. Even though the association between HLA-B27 and ReA is known for decades, the exact mechanism how HLA-B27 confers the disease susceptibility is still not known. However, it has been suggested that the interaction between HLA-B27-positive host cells and disease-triggering bacteria is disturbed, leading to an inefficient elimination of causative bacteria. In HLA-B27-expressing cells the triggering bacteria are known to survive longer than in control cells. Furthermore, it was discovered that bacteria are able to replicate inside HLA-B27-expressing cells. Interestingly, this feature seems to be associated with the tendency of HLA-B27 heavy chains (HCs) to misfold. In fact, one of the theories proposed to explain the role of HLA-B27 in the pathogenesis is HLA-B27 misfolding hypothesis. It suggests that the disease results from the accumulation of aberrantly folded HLA-B27 HCs in the endoplasmic reticulum (ER) causing ER stress. The activation of the ER response pathway could lead to an inflammatory response and release of proinflammatory cytokines contributing to disease pathogenesis.

Our research group´s resent results indicate that the expression of HLA-B27 molecule alters the signaling molecules and/or signaling pathways, which are involved in inflammatory reactions. The p38 mitogen-activated protein kinase (MAPK) signaling pathway, which is important in regulating the replication of *Salmonella enteritidis* in human monocytic U937 cell line cells, does not function properly in HLA-B27-expressing cells. Furthermore, double-stranded RNA activated kinase (PKR), which is an ER stress responsive kinase, is overexpressed but hypophosphorylated in HLA-B27-expressing U937 cells. These facts prompted us to study the signaling pathways further in HLA-B27-expressing cells.

This study was designed to reveal whether the expression of HLA-B27 molecule modifies the activation of such signaling molecules and/or signaling pathways, which are involved in inflammatory reactions. Better understanding of the mechanism how HLA-B27 causes susceptibility to SpA diseases will help to develop more specific and effective treatment against ReA and other SpAs in future.

2. REVIEW OF LITERATURE

2.1 Spondyloarthritis

Spondyloarthritides (also known as spondyloarthropathies, SpAs) are a group of chronic, inflammatory rheumatic diseases, which share several common clinical symptoms and epidemiologic and genetic factors. They are usually characterized by the involvement of inflammation in sacroiliac joints, peripheral inflammatory arthropathy and insertional tendinitis (reviewed in Armas et al., 2009). SpAs are divided into the subgroups, which are ankylosing spondylitis (AS), reactive arthritis (ReA), psoriatic arthritis (PsA), entero-associated arthritis (EA) such as Crohn´s disease and ulcerative colitis and undifferentiated spondyloarthritis (uSpA). According to some definitions also juvenile SpA (JSpA) and arthritis associated with acute anterior uveitis are included in SpAs (reviewed in Bakland and Nossent, 2013; van den Berg and van der Heijde, 2010 and Wittoek and Mielants, 2009). The Assessment of SpondyloArthritis International Society (ASAS) divides SpAs further in to two groups: predominantly peripheral SpA, which includes ReA, PsA, EA and uSpA and predominantly axial SpA, which includes AS and early, nonradiography axial SpA (Rudwaleit et al., 2009).

The estimation of SpAs prevalence is especially challenging due to the variations in diagnostic and the methodology of surveys. However, diseases belonging to the group of SpA are estimated to affect around 1 % of world's population (reviewed in Colbert et al., 2014). In the USA AS and/or SpAs are affecting approximately 0.4-1.3 % of the population, whereas in Finland in 1995 the estimated incidence rate was 6.9 per 100 000 person a year (reviewed in Bakland and Nossent, 2013; Kaipiainen-Seppänen and Aho, 2000).

Since the heterogeneous nature of SpA diseases, there is no single distinguishing feature or symptom shared by all SpAs. The most frequent symptom specified is back pain. Symptoms such as oligoarthritis (predominantly of the lower limbs), dactylitis, enthesitis at heel or other sites, and extra-articular manifestations such as uveitis, inflammatory bowel disease and psoriasis can all be present in each of the SpA subgroups. In addition, bone loss is more and more recognized finding in SpAs (reviewed in Anandarajah and Schwarz, 2009; Rojas-Vargas et al., 2009; reviewed in van Tubergen and Weber, 2012). According to ASAS classification criteria, peripheral arthritis, enthesitis, especially heel enthesitis, and dactylitis are present in about 25 % of SpA cases. ASAS also lists some extra-articular symptoms like anterior uveitis (23 %), psoriasis (23 %), and inflammatory bowel disease (IBD) such as Crohn´s disease

and colitis ulcerosa (17%) to be associated with SpAs (reviewed in van den Berg and van der Heijde, 2010).

The tendency towards familial aggregation is well known in SpAs. The genetic association of SpAs to human leucocyte antigen (HLA)-B27 was first described in 1973 (Aho et al., 1973; Brewerton et al., 1973; Schlosstein et al., 1973). The association of HLA-B27 was first discovered with AS (Brewerton et al., 1973). It was also the first association described between HLA alleles and inflammatory diseases. Furthermore, the association of HLA-B27 with AS is one of the strongest known association between diseases and HLA molecule. Almost 90 % of patients suffering from AS are HLA-B27-positive (reviewed in Hannu, 2011 and McHugh and Bowness, 2012)).

The strongest evidence of involvement of HLA-B27 in SpA is provided by animal studies with HLA-B27 transgenic rats. Several transgenic rat lines carrying either human HLA-B27 or human β_2-microglobulin (a component of major histocompatibility complex (MHC) class I molecules), develop a spontaneous multisystemic inflammatory arthritic disease, closely resembling human SpA (reviewed in Antoniou et al., 2011; Hammer et al., 1990; Taurog et al., 1999).

2.1.1 Reactive arthritis

The term "reactive arthritis" was first used in 1969 (Ahvonen et al., 1969). It was used to describe conditions following infection elsewhere in the body, but in which no microbial antigens could be isolated from the joint (Ahvonen et al., 1969). Traditionally, ReA is described as nonseptic arthritis which develops during or soon after bacterial infection in the gastrointestinal or urogenital tract, but in which the micro-organism does not enter the joint cavity (Aho et al., 1973). Today, it has been proved that antigens of triggering microbes can be detected in the synovial tissues of affected joints. Bacterial antigens such as lipopolysaccharide (LPS) and heat shock proteins are found in synovial fluids or synovial cells of inflamed joints (Granfors et al., 1990; Granfors et al., 1992; Granfors et al., 1989). In some cases, bacterial deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), from *Chlamydia* and *Yersinia* for example, are detected in inflamed joints (Gaston et al., 1999; Gérard et al., 1998; Gérard et al., 2000).

ReA-triggering bacteria have certain common characteristic features; they are facultative or obligate intracellular gram-negative (they have a LPS) bacteria, which cause mucosal infections (Granfors, 1992). The most common ReA-triggering bacterium is *Chlamydia trachomatis*, which causes genitourinary track infections (Rich et al., 1996). In addition, enteropathogenic bacteria such as *Salmonella, Yersinia, Shigella and Campylobacteri* species are common to trigger ReA (reviewed in Hannu, 2011). There are also some case reports of atypical ReA-triggering bacteria like *Escherichia*

coli (Laasila and Leirisalo-Repo, 1999) *Chlamydia pneumonia* (Braun et al., 1994), *Clostrium difficile* (Jacobs et al., 2001) and *Giardia lablia* (Cantey et al., 2011; Layton et al., 1998).

On average 1-4 % of the people with potentially causative agents develop ReA after infection. It usually affects young adults aged 20-40 year. The frequency among males and females is almost the same after gastrointestinal infection, whereas *C. trachomatis* triggered ReA is more common in males (reviewed in Hannu, 2011; Kwiatkowska and Filipowicz-Sosnowska, 2009). The symptoms of ReA

usually develop within two or three weeks of the preceding infection and the typical duration of arthritis is four to five months. However, 15-30 % of the patients develop chronic or recurrent peripheral arthritis, sacroilitis and/or spondylitis (reviewed in Khan, 2002). The most common symptom of ReA is an acute asymmetric and oligoarticular inflammation affecting mainly the larger joints of lower limps. Patients may also suffer from inflammatory back pain and sacroilitis in addition to some other characteristics extra-articular features such as ocular inflammation, dactylitis and enthesitis (like heel pain, which is typical of ReA) (reviewed in Hannu, 2011 and Kwiatkowska and Filipowicz-Sosnowska, 2009).

The population –based studies estimate the annual occurence of ReA to be 0.6-27/100 000. The incidence rate varies greatly among different geographic locations, but also how ReA is defined. In the arthritis survey done in Finland at the year 2000, 10 cases of ReA/100 000 were reported (reviewed in Hannu, 2011; Savolainen et al., 2003).

As all SpAs, also ReA has a strong association with the HLA-B27 (Aho et al., 1974). As much as 80 % of the patients with ReA carry the HLA-B27 antigen. The presence of HLA-B27 seems to be associated especially with more severe forms of arthritis and predicts longer disease duration. However, outbreak studies or epidemiological surveys at population level show only a slight or no increased frequency of HLA-B27 (reviewed in Hannu, 2011).

2.2 HLA-B27

Human leucocyte antigen (HLA)-B27, encoded within the HLA-B locus, was first discovered in 1969 (Thorsby, 1969). HLA-B27 is MHC class I cell surface molecule. It is a multisubunit glycoprotein, consisting of polymorphic MHC I-encoded heavy chains (HC), β_2-microglobulin (β_2m) and a small peptide (usually 8-10 amino acid residues long). HLA-B27 is constructed in the endoplasmic reticulum (ER) (reviewed in Colbert, 2004) (Figure 1.).

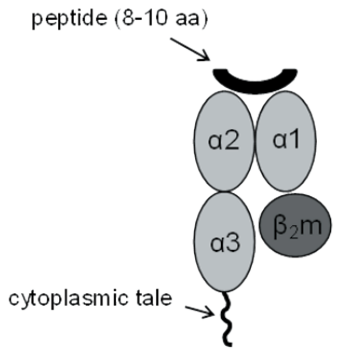

Figure 1. Structure of MHC class I molecule. MHC class I molecule consists of a heavy chain with three polypeptide domains (α1-α3). The β2 microglobulin is covalently attached to the heavy chain.

Only 4 years after HLA-B27 was discovered, it was found to have a remarkable association with AS and SpAs (Aho et al., 1973; Brewerton et al., 1974; Brewerton et al., 1973; Schlosstein et al., 1973). The associations of HLA-B27 with different subtypes of SpAs are presented in table 1 (reviewed in Khan, 2002 and Penttinen et al., 2004a) (Table 1.). To date, there are at least 105 known HLA-B27 subtypes encoded by 132 different alleles. Subtypes are named HLA-B*27:01 to HLA-B*27:106. They differ from each other, based on their amino acid sequence, usually only by one or few amino acids. The most widely distributed subtype is HLA-B*27:05; which is also considered the ancestral allele from which all the other subtypes are evolved. Subtypes, which are most frequently associated with SpAs are HLA-B*27:02, HLA-B*27:04 and HLA-B*27:05, whereas for example subtypes HLA-B*27:06 and HLA-B*27:09 appear to lack the disease association (reviewed in Khan, 2013).

Table 1. Subgroups of spondyloarthritis diseases, and their association with HLA-B27 in white persons. Modified from Khan, 2002.

Disease	Abbreviation	Prevalence of HLA-B27 app. %
Ankylosing spondylitis	AS	90
Reactive arthritis	**ReA**	**40-80**
Psoriatic arthritis	PsA	40-50
Entero-associated arthritis	EA	35-75
Undifferentiated spondyloarthritis	uSpA	70
Juvenile SpA	JSpA	70
Arthritis with acute anterior uveitis		50

HLA-B27 is virtually expressed on the cell surface of all nucleated cells. As MHC class I molecule its main function is to present intracellular antigens to CD8+ T cells on the cell surface. Peptides derived either from intracellular pathogens or self-proteins are processed in proteasomes and transported to the ER. In the ER, they are assembled with heterodimeric complexes which contains the heavy chains of MHC class I molecule and β_2-microglobulin. The complete peptide-MHC I-complexes are then transferred to the cell membrane where they present antigens to specific class I restricted CD8+ T cells (reviewed in Pamer and Cresswell, 1998). The most dominant effect on peptide selection in HLA-B27 molecule resides in the B pocket, a region in the peptide binding groove of HC. Interestingly, certain amino acids such as histidine at position 9, glutamic acid at position 45, cysteine at position 67, lysine at position 70, alanine at position 71 and glutamine at position 97, in the B pocket of the HC are highly conserved among the disease-associated subtypes of HLA-B27, suggesting that these residues may be important in the development of pathogenesis (reviewed in Breban et al., 2004).

HLA-B27 molecule possesses some unusual features. First, its HC has an unusually slow folding rate, which can easily lead to formation of misfolded HLA-B27 HC. Second, HLA-B27 is able to form aberrant disulfide-linked dimers on cell surface (reviewed in Colbert et al., 2014). Both of these features are linked to the molecule composition of the B pocket. Mutations of certain amino acid residues in the B pocket are shown to enhance the folding kinetics and prevent the misfolding of the HC (Mear et al., 1999). For example, a single amino acid mutation of glutamic acid to methionine at position 45 in the B pocket of the HC prevents the misfolding of HLA-B27 molecule. A change of six amino acids in the B pocket (H9F, T24A, E45M, I66K, C67V and H70K) leads to the resemblance of the B pocket of another MHC class I molecule, HLA-A2, which, does not misfold (Dangoria et al., 2002; Mear et al., 1999). In addition, an unpaired and reactive cysteine residue (Cys67) in the B pocket forms disulphide links between two HLA-B27 HCs allowing them to form dimers (Dangoria et al., 2002). The third unusual feature of HLA-B27 molecule is its peptide binding specificity. The peptide binding specificity is linked to HLA-B27 subtypes, it has been speculated that this could be a critical feature determining the pathogenic role of HLA-B27, either by presenting arthrogenic peptides differently or by modulating other molecular features such as folding assembly and export (reviewed in de Castro, 2009).

2.3 Theories of HLA-B27 and pathogenesis

Although the exact mechanism how HLA-B27 confers to disease susceptibility is still not known, several theories have been proposed to explain HLA-B27s role in the pathogenesis. Some of these theories are based on HLA-B27 function in antigen

presentation; whereas others are based on atypical properties of HLA-B27 (like misfolding and cell surface dimerization). Three of the main theories, which are still under investigation are: the arthritogenic peptide hypothesis, the HLA-B27-free heavy chain and homodimer hypothesis, and the HLA-B27 misfolding and unfolded protein response (UPR) hypothesis (reviewed in McHugh and Bowness, 2012).

The arthritogenic peptide theory is one of the first and most studied hypothesis to explain the role of HLA-B27 in the disease. It is based on the antigen presentation function of HLA-B27. It proposes that HLA-B27 binds and presents arthritogenic peptides derived from the disease-triggering bacteria which are cross-reactive with the self-peptides in the joint. This would lead activation of CD8+ cytotoxic T-cells (CTLs) to recognize self-peptides in the joint and initiate autoimmune response (reviewed in Benjamin and Parham, 1990 and McHugh and Bowness, 2012). Endoplasmic reticulum aminopeptidase 1 (ERAP1), an aminopeptidase enzyme, which trims peptides in the ER to optimal length for HLA class I molecule binding, is recently associated with AS and HLA-B27 (Harvey et al., 2009). It has been speculated that changes in ERAP1 function may alter the selection of peptides available to bind to HLA-B27 and thus for example, alter the presentation of arthritogenic peptides on cell surface (reviewed in McHugh and Bowness, 2012).

The second theory, the HLA-B27-free HC and homodimer hypothesis, is based on aberrantly folded cell surface HLA-B27 molecules. A trigger, like bacterial infection or stress-related signal may promote the formation of β_2m-free or peptide free HLA-B27 HCs to form HC monomers or HC dimers on cell surface from unstable HLA-B27 precursors. The dimers are stabilized by disulphide bond between the free cysteine residues at position 67 in the B pocket (Allen et al., 1999; Dangoria et al., 2002). These dimers probably arise from a recycling of fully folded HLA-B27 cell surface molecules through the endocytic pathway (Bird et al., 2003). The aberrantly folded cell surface HLA-B27 monomers and dimers are then recognized by innate immunoregulatory receptors on antigen presenting cells, like macrophages and dendritic cells, which may lead to a disturbed signaling in these cells and cause proinflammatory responses (reviewed in McHugh and Bowness, 2012). In addition to HLA-B27 dimerization on the cell surface, HLA-B27 HC may form aberrant disulfide complexes during assembly in the ER (Dangoria et al., 2002). This will be discussed more with the HLA-B27 misfolding hypothesis.

The third theory, the HLA-B27 misfolding hypothesis, suggests that the disease results from the accumulation of aberrantly folded HLA-B27 in the ER, causing the inflammatory response (reviewed in Colbert et al., 2009; Mear et al., 1999). Misfolding of HLA-B27 is discussed in more detail below.

2.3.1 HLA-B27 misfolding in the ER

As stated above, HLA-B27 has a tendency to misfold during assembly in the ER. Normally, MHC class I molecules are synthesized, folded and assembled in the ER with the help of ER resident helper proteins called chaperones. Chaperones such as calnexin, calreticulin, Erp75, tapasin and transporter associated with antigen processing 1 (TAP1) and TAP2 help MHC encoded HC, β_2m and 8-10 amino acid long peptide to accomplish a mature, properly folded and assembled structure. Properly folded three subunit structures are transported from the ER to the cell surface through Golgi machinery (reviewed in Van Hateren et al., 2010) (Figure 2.). Incorrectly assembled HCs are retranslocated in the cytosol and degraded by proteasomes in the ER-associated degradation process (ERAD) as part of a quality control process (reviewed in Colbert et al., 2014). However,

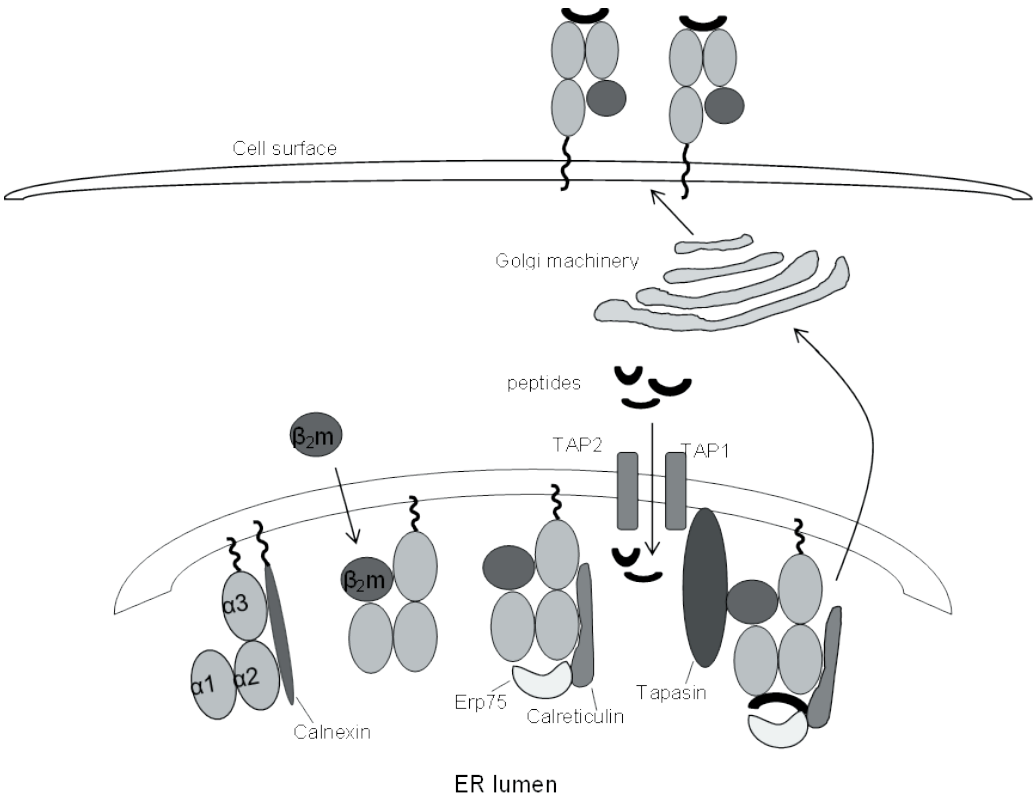

Figure 2. The assembly of HLA-B27 molecules in the ER. HLA-B27 molecule is assembled in the ER with the help of several chaperone molecules to achieve mature, properly folded and assembled structure. Calnexin assists alpha domains of the heavy chain to fold in the membrane of the ER. After HC folding is completed and β2-microglobulin is attached, calnexin is replaced by calreticulin and Erp75. Erp75 helps the HCs disulfide bond formation. Two subunits of TAP protein, TAP1 and TAP2, function as a transporter for the peptides from cytosol to the ER. Tapasin recruits HLA-B27 molecules to the TAP protein, and together with TAP it enables the peptide loading to HLA-B27 molecule. Properly folded HLA-B27 molecules are transported to the cell surface via Golgi machinery. Adapted from Van Hateren et al., 2010 and Colbert et al., 2004.

misfolded proteins may accumulate in the ER and trigger a stress response. There are two types of probably partially overlapping ER stress responses, UPR and ER overload response (EOR) (reviewed in Colbert, 2004). These stress responses will disturb normal cellular functions and eventually lead to production of proinflammatory cytokines, which may promote the development of SpAs (reviewed in Antoniou et al., 2011). The misfolded proteins may also escape the quality control process and traffic to other cellular compartments or to cell surface (Mear et al., 1999).

Evidence of HLA-B27 molecule to misfold is based on several findings. One of them is the occurrence of ERAD, a process where misfolded proteins are transferred from the ER to cytosol to be degraded by the proteasomes (reviewed in Colbert et al., 2014; Mear et al., 1999). Among other events, ERAD process involves the ubiquitination of the proteins to be degraded. This requires the E3 ubiquitin ligase, HMG-CoA reductase degradation 1 (HRD1). Interestingly, the absence of HRD1 causes accumulation of the misfolded HLA-B27 oligomers, even with the presence of normal MHC class I assembly pathway (Burr et al., 2011). Other evidence for HLA-B27 misfolding comes from the formation of oligomer structures like dimers: HLA-B27 HCs forms aberrant disulphide-linked complexes during the folding and assembly process in the ER. The formation of these oligomers is associated with the cysteine residue 67 on the B pocket of HLA-B27, although a structurally conserved cysteine at position 164 seems to be also involved. In the absence of ER chaperone molecule tapasin or β_2m, HLA-B27 HC dimers start to accumulate in the ER (Dangoria et al., 2002). These dimer constructs do not traffic to the cell surface through the secretory pathway, and the dimerization in the ER is distinctive from the cell surface dimerization (Antoniou et al., 2004; Dangoria et al., 2002). The misfolding of HLA-B27 molecule is further supported by the prolonged binding of immunoglobulin heavy-chain binding protein (BiP). BiP is an ER chaperone molecule, which normally binds transiently to newly synthesized ER proteins. However, HLA-B27 HC is shown to be more prone to bind with BiP and exhibit prolonged association with the molecule (Tran et al., 2004). BiP is also a major regulator of UPR. In unstressed cells, BiP functions as a repressor by binding to the ER luminal domains of the main UPR effector proteins. However, in stressed cells, BiP binds to accumulating misfolded or unfolded proteins instead. The released UPR effector proteins are then allowed to activate nuclear events downstream (reviewed in McHugh and Bowness, 2012).

The accumulation of misfolded HLA-B27 HCs in the ER can cause a strong and chronic stimulation of the ER stress response pathways, which in turn can lead to a release of proinflammatory cytokines contributing to disease pathogenesis. This hypothesis is supported by transgenic animal studies (Tran et al., 2004; Turner et al., 2005). HLA-B27 transgenic rats prone to have a disease are shown to have misfolded HLA-B27 HC accumulated in their macrophages and splenocytes (Turner et al., 2005).

Furthermore, misfolded HLA-B27 molecules in HLA-B27 transgenic rats are more apt to bind BiP (Tran et al., 2004). The misfolding theory may also offer an explanation why some subtypes are associated more strongly with the disease, since alleles, which are more strongly associated with the disease may have an increased tendency to misfold (reviewed in McHugh and Bowness, 2012).

2.4 Interaction between HLA-B27 and ReA-triggering bacteria

Several studies show evidence that the interaction between HLA-B27-positive host cells and ReA-triggering bacteria is disturbed and the elimination of causative bacteria is inefficient (Kapasi and Inman, 1992; Laitio et al., 1997; Penttinen et al., 2004b; Saarinen et al., 2002; Virtala et al., 1997). For example, the expression of HLA-B27 is shown to enhance the intake of ReA-triggering bacteria in intestinal epithelial cells (Saarinen et al., 2002). On the other hand, in mouse L cell fibroblasts and cultured fibroblasts the expression of HLA-B27 is observed to inhibit the invasion of many ReA-triggering bacteria (Kapasi and Inman, 1992, 1994; Virtala et al., 1997).

Although the uptake of *Salmonella enteritidis* in monocytes is shown to be unaffected by the expression of HLA-B27 (Laitio et al., 1997), the elimination of *S. enteritis* is impaired in HLA-B27-transfected cells compared with control cells (Laitio et al., 1997; Virtala et al., 1997). More detailed studies revealed that the HLA-B27-expressing cells are actually more permissive for the intracellular replication of *S. enteritidis* than the control cells (Penttinen et al., 2004b). This phenotype appears to be dependent on the HLA-B27 misfolding, since in cells, which express mutated forms of HLA-B27 HC, and which do not misfold anymore, the replication of intracellular *S. enteritidis* was not evident (Penttinen et al., 2004b). In addition, over a 10-fold increase has been reported in the number of live bacteria in HLA-B27-positive human fibroblasts compared with HLA-B27-negative cells 7 days after infection (Huppertz and Heesemann, 1996).

Several contradictory studies, however, indicate that HLA-B27 does not influence the elimination or survival of ReA-triggering bacteria (Huppertz and Heesemann, 1997; Saarinen et al., 2002; Young et al., 2001). Huppertz and Heesemann, 1997 reported that either the invasion or the replication of *S. enteritidis* were not affected by HLA-B27 in human fibroblasts (Huppertz and Heesemann, 1997). Moreover, the survival of *Salmonella* or *Yersinia* was not influenced by the expression of HLA-B27 in intestinal epithelial cells (Saarinen et al., 2002). Young et al, 2002 reported that HLA-B27 influences neither the infection nor replication of *C. trachomatis* in B cell line cells (Young et al., 2001).

2.5 STAT1

The family of signal transducers and activators of transcription (STAT) has seven members: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6. They were first discovered in early 1990´s as key proteins in cytokine signaling. As their name implies, they transduce signals from the cell membrane to the nucleus to activate gene transcription (Fu et al., 1992; Schindler et al., 1992). STATs are involved in the regulation of cell growth, cell differentiation, cell survival, and in the cross-talk between immune and cancer cells. Furthermore, STATs have numerous functions in innate immunity, especially STAT1, which is a key mediator of the interferon (IFN)-γ signaling pathway (reviewed in Santos and Costa-Pereira, 2011).

STAT1 was first described as a mediator of IFN-α signaling (Levy et al., 1989), but later STAT1 was discovered to be an important player in the cellular response to IFN-γ (Meraz et al., 1996). In addition to interferons a wide variety of other cytokines can activate STAT1, including interleukins, growth factors and hormones. STAT1 participates in various cellular events, such as growth inhibition, apoptosis and tumor suppression (reviewed in Lim and Cao, 2006). Moreover, STAT1 is known to have indispensable role in immune response to bacteria, viruses and parasites (Dupuis et al., 2003; Durbin et al., 1996; Gavrilescu et al., 2004; Horvath and Darnell, 1996; Rosas et al., 2003). The IFN-induced phosphorylation of STAT1 is responsible for the activation of macrophages. Furthermore, STAT1 is shown to be important in the development of systemic inflammation (Herzig et al., 2012). Interestingly, increased STAT1 expression and activation is seen in the intestine of the patients with IBD, particularly in ulcerative colitis (Schreiber et al., 2002). However, it has been shown that in peripheral blood dendritic cells derived from Crohn´s disease patients, IFN-α induced STAT1 phosphorylation is impaired in (Nieminen et al., 2013).

The reduced activity of STAT1 is associated with susceptibility to many infectious diseases. STAT1-deficient mice are highly sensitive to infection caused by microbial pathogens and viruses and they die mainly on viral infection in less than eight weeks (Durbin et al., 1996; Meraz et al., 1996). In humans, mutations which interfere with the function of STAT1 are associated with high sensitivity to mycobacterial infections (Chapgier et al., 2006; Chapgier et al., 2009). STAT1 deficiency is also shown to increase the risk of invasive salmonellosis in humans (Averbuch et al., 2011). Furthermore, the high sensitivity to viral diseases is shown to be associated with such mutations in the STAT1 gene, which leads to complete absence of the protein (Dupuis et al., 2003). It has been shown that STAT1-deficient mice are resistant to LPS-induced shock as well (Karaghiosoff et al., 2003). In addition, the systemic inflammation is attenuated in STAT1-deficient mice (Herzig et al., 2012). Interestingly, it has been suggested that STAT1 controls joint inflammation in mice, since STAT1 deficiency aggravated the symptoms of chronic joint inflammation (de Hooge et al., 2004).

2.5.1 Structure of STAT1

STAT proteins share several highly homologous regions. These regions are the N-terminal domain (NTD), the coiled-coil domain (CCD), the DNA binding domain (DBD), the linker domain (LD), the Src Homology 2 (SH2) domain and the C-terminal transactivation domain (TAD) (Figure 3). The N-terminal domain of STAT1 is important for the dimerization of unphosphorylated STAT monomers. It is also important for the nuclear import and deactivation of STAT1. Next to the NTD is the coiled-coil domain, which is involved in the interaction with other proteins. CCD also contains a nuclear export signal (NES) of STATs. The DNA binding domain is highly conserved among STATs. This domain is involved in the nuclear translocation of activated STAT1. The α-helical linker domain links the DBD to SH2, and is important for the transcriptional activation of STAT1 in response to IFN-γ. The most conserved region of STATs is SH2 domain. It is necessary for the receptor association and formation of tyrosine phosphodimers, since the phosphorylation of STAT1 tyrosine 701 residue and subsequent homodimerization are dependent on the residues located in SH2 domain. The C-terminal end of STAT1 functions as a transactivation domain, and the key phosphorylation sites of STAT1, tyrosine 701 and serine 727 are located in the C-terminal area (reviewed in Levy and Darnell, 2002 and Santos and Costa-Pereira, 2011).

STAT1 has two isoforms as a result of alternative messenger RNA (mRNA) splicing. The STAT1α isoform is full-length and it is 91 kD long. The shorter isoform, STAT1β, is truncated at the C-terminal end of TAD and is 84 kD long. (Figure 3.) As a consequence of the truncation of the last 38 amino acids of the C-terminal end, STAT1β isoform lacks the serine 727 phosphorylation site. Therefore, the activation, transcriptional activity and biological functions of STAT1α and STAT1β isoforms differ from each other slightly (Schindler et al., 1992).

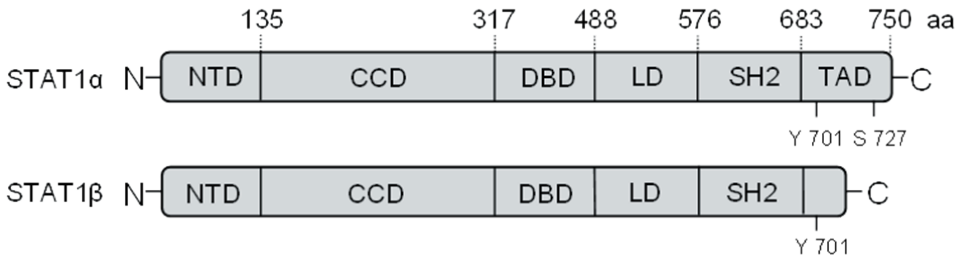

Figure 3. Structure of STAT1. Structure of STAT1 isoforms, STAT1α and STAT1β. NTD = N-terminal domain, CCD = coiled-coil domain, DBD = DNA-binding domain, LD = linker domain, SH2 = SH2 domain and TAD = C-terminal transactivation domain.

2.5.2 JAK/STAT1 signaling pathway

In the classical JAK-STAT1 pathway, the cytokine binding to a receptor leads to dimerization or oligomerization of the receptors and subsequent activation of the receptor-associated tyrosine kinases, Janus kinases (JAK). Activated JAKs then phosphorylate the tyrosine residues of the cytoplasmic tail of the receptor, which provides a docking site for the latent cytoplasmic STAT1. STAT1 binds to the JAK via SH2 domain. Next, JAKs phosphorylate STAT1 on tyrosine 701 residues on the C-terminal end. Tyrosine phosphorylated STATs are then able to form homo- or heterodimers via phosphorylated tyrosine residue of the one partner to SH2 domain of the other. Finally, STAT dimers are released from the receptor and translocated to the nucleus by importin molecules. In the nucleus STAT1 dimers bind to specific DNA binding elements, IFN-stimulatory element (ISRE) and IFN-γ activation site (GAS) response elements, to activate the gene transcription (reviewed in Adámková et al., 2007 and Lim and Cao, 2006) (Figure 4.).

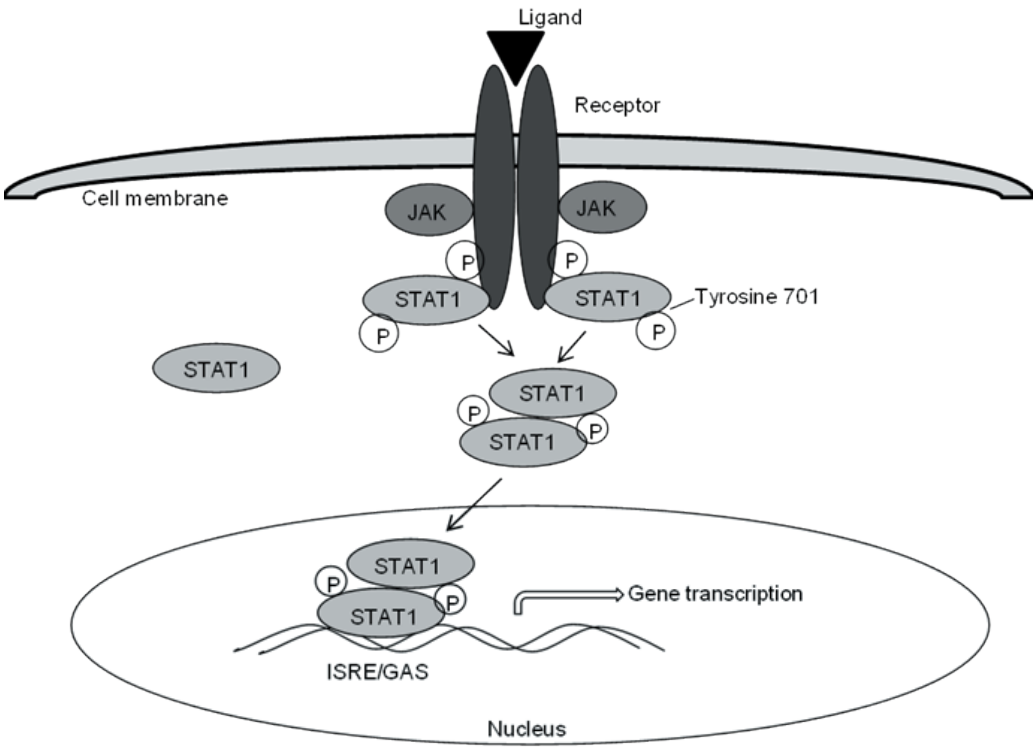

Figure 4. Activation of the JAK-STAT1 pathway. Ligand binding to a receptor results receptor dimerization and activation of JAKs. Activated JAKs bind and phosphorylate the cytoplasmic tyrosine residues on the receptor. Phosphorylated tyrosine residues act as docking sites for latent STAT1, and JAKs are able to phosphorylate STAT1 in the tyrosine residue. Phosphorylated STATs form dimers, which are translocated to the nucleus, and bind to DNA activating gene transcription. Adapted from Adámková et al., 2007 and Lim and Cao, 2006.

The activation of gene transcription is fairly fast, since translational response can be detected within a few minutes after ligation binding to a receptor (reviewed in Ivashkiv and Hu, 2004). The maximum accumulation of the phosphorylated STAT1 in the nucleus is seen by 30 minutes and the half life of the phosphorylated STAT1 is less than 15 minutes (Haspel et al., 1996). After STAT1 is released from DNA it is dephosphorylated and transported back to the cytoplasm. This involves interaction of the nuclear export sequence of the STAT1 with the export molecule, exportin-1, or other export molecules (reviewed in Lim and Cao, 2006). Two molecules which are known to dephosphorylate and inactivate STAT1 are nuclear T-cell protein tyrosine phosphatase TC45 and SH2-domain containing protein tyrosine phosphatase 2 (SHP-2) (ten Hoeve et al., 2002; Wu et al., 2002).

2.5.3 Activation of STAT1

Interferons are the main activators of STAT1. It has been shown that both type I and type II interferons, IFN-α/β and IFN-γ, activate STAT1. IFNs are important in macrophage activation and host defense in response to microbial pathogens (Durbin et al., 1996; Meraz et al., 1996). Mice lacking STAT1 are highly susceptible to microbial and viral infections as well as tumor formation because of the severely impaired IFN-γ and IFNα/β responses (Durbin et al., 1996). Interestingly, certain cytokines are known to preferentially activate some particular STATs, and IFN-γ is known to especially activate STAT1. IFN-γ is one of the most important endogenous mediators in immunity and inflammation. Many of the IFN-γ functions are, in fact, mediated by STAT1. STAT1 is shown to directly activate many immune effector genes, like genes which encodes microbicidal molecules, antiviral proteins, phagocytic receptors, cytokines, chemokines and antigen presenting molecules (reviewed in Hu and Ivashkiv, 2009). IFN-γ is also able to upregulate MHC class I expression on the cell surface. The MHC class I genes are regulated by Nuclear Factor kappa B (NF-κB) and Interferon regulatory factor 1 (IRF-1) via IFN-γ-mediated JAK/STAT1 signal transduction pathway (reviewed in Zhou, 2009).

In addition to interferons, STAT1 is also activated in response to several other cytokines, such as interleukin (IL)-6 and IL-2, growth factors, like epidermal growth factor (EGF), platelet derived growth factors (PDGF) and others, which respond to receptors with intrinsic tyrosine kinase activities (reviewed in Lim and Cao, 2006; Najjar and Fagard, 2010 and Subramaniam et al., 2001). Some oncoproteins, like v-src and Latent membrane protein 1 (LMP1) are known constitutively activate STAT1 (Cirri et al., 1997; Gires et al., 1999). In addition, oncostatin M, growth hormones, angiotensin II and tumor necrosis factor (TNF) can activate STAT1, although the two latter ones appear

to activate STAT1 in cytoplasmic way (Chauhan et al., 1995; Guo et al., 1998; Marrero et al., 1995; Smit et al., 1996).

2.5.4 Phosphorylation of STAT1

The activity of STAT1 is regulated by phosphorylation. It has two major phosphorylation sites: tyrosine residue at position 701 and serine residue at position 727. They are both located in the C-terminal end of the protein, although, the STAT1β isoform lacks the serine 727 phosphorylation site because of the C-terminal splicing of STAT1 (reviewed in Najjar and Fagard, 2010) (Figure 3.).

The main phosphorylation site of STAT1 is tyrosine 701. It becomes phosphorylated in response to various stimuli engaging the cell surface receptors. Interferons are the main activators of STAT1 but also some interleukins, growth factors, oncoproteins and growth hormones are able to induce STAT1 tyrosine 701 phosphorylation (reviewed in Lim and Cao, 2006). Fascinatingly, the phosphorylation of this single tyrosine 701 residue of STAT1 is shown to be sufficient to induce STAT1 dimerization, nuclear localization and DNA binding (Shuai et al., 1993; Wen et al., 1995). For example, Shuai et al, 1993 demonstrated that the phosphorylation of tyrosine 701 is required for nuclear translocation, DNA binding and IFN-γ induced gene transcription in STAT1. STAT1β (missing serine 727 residue) was able to translocate to the nucleus and bind DNA as well, although gene transcription was not observed (Shuai et al., 1993). In addition, STAT1 carrying mutation in serine 727 site is shown to phosphorylate normally on tyrosine 701 residue, dimerize and bind DNA, even though the transcriptional efficiency is decreased in this case too (Wen et al., 1995).

The other important phosphorylation site of STAT1 is serine 727. It has been reported that the phosphorylation of this site is needed for the maximal transcriptional activity of STAT1 (Wen et al., 1995). The phosphorylation of STAT1 serine 727 site is induced by several serine kinases, including extracellular-signal-regulated kinases 1 and 2 (ERK1/2), p38 mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinases (JNK), protein kinase C δ (PKCδ) and $Ca2^{+}$/calmodulin-dependent protein kinases II (CAMKII). In addition, various stress conditions like bacterial infection, bacterial LPS and ultra violet (UV) irradiation are known to trigger the serine 727 phosphorylation of STAT1 (reviewed in Lim and Cao, 2006 and Najjar and Fagard, 2010). The importance of STAT1 serine 727 phosphorylation is proved in mutation studies. Only about 20 % of IFN-γ-dependent transcription was observed in cells after the mutations of STAT1 serine 727 to alanine (S727A) (Wen et al., 1995). Furthermore, mice expressing a S727A mutation of STAT1 are shown to be highly sensitive to bacterial infections. These mice have also strongly reduced IFN-γ-induced gene expression (Varinou et

al., 2003). The important role of STAT1 serine 727 residue mediating apoptosis is reported as well. Cells carrying S727A mutation in STAT1 protein are shown to be more resistant to TNF or actinomycin D induced apoptosis than the wild type cells (Kumar et al., 1997).

The phosphorylation of serine 727 can also happen independently of STAT1 tyrosine 701 phosphorylation (Wen and Darnell, 1997). As an example, in macrophages, the stress-induced STAT1 serine 727 phosphorylation in response to UV irradiation, LPS or TNF occurs through the p38 MAPK signaling pathway; independently of tyrosine 701 phosphorylation (Kovarik et al., 1999; Kovarik et al., 1998).

It is increasingly acknowledged that also unphosphorylated STATs are transcriptionally active and they are able to continuously shuttle between the nucleus and the cytoplasm (reviewed in Meyer and Vinkemeier, 2004). In addition, it has been shown that unphosphorylated nuclear STAT1 is able to prolong the IFN-induced transcription of regulatory genes which are involved in immune responses (Cheon and Stark, 2009).

2.5.5 Other regulatory mechanism of STAT1

In addition to phosphorylation, there are several other mechanisms by which the activity of STAT1 can be regulated. Many of them are post-translational modifications such as ubiquitination, ISGylation (ISG,interferon stimulated gene), SUMOylation (SUMO, small ubiquitin-like modifier), and methylation (reviewed in Lim and Cao, 2006).

Ubiquitination is a mechanism by which proteins are targeted to degradation via proteasome pathway. Interestingly, one of the ubiquitin enzyme ligands, skeletal muscle LIM protein (SLIM), is shown not only to cause STATs proteosome-mediated degradation, but also to enhance the dephosphorylation of the tyrosine residue of STATs (at least in STAT4) (Tanaka et al., 2005). STAT1 is the only STAT protein, which is targeted to SUMOylation. Although the different SUMO proteins seem to SUMOylate STAT1 differently, and the physiologic function of STAT1´s SUMOylation is not known, it has been proposed that SUMOylation is involved in the negative regulation of certain STAT1-mediated transcriptional responses (Ungureanu et al., 2005). Like SUMOylation, ISGylation is restricted to STAT1 proteins among STAT family, although its biological significance is still not known. However, there is indication that ISGylation could have a positive regulatory role on STAT1 tyrosine 701 phosphorylation (Malakhova et al., 2003). The meaning of methylation of STAT1 is also still controversial, but some models propose that it increases the DNA binding activity and gene transcription of STAT1 (Mowen et al., 2001).

SOCS (suppressor of cytokine signaling) and PIAS (protein inhibitor of activated STAT) proteins are negative regulators of STATs. SOCS proteins inhibit the phosphorylation of STATs by interfering with the activation of JAKs or by competing with STATs for binding to cytokine receptors. SOCS are constantly expressed in low levels, but their expression is induced by cytokines and bacterial cell wall structures like LPS. Also activated STATs can induce the expression of SOCS as a negative feedback mechanism. Although SOCS proteins have no specificity for certain STAT protein, they are specific for certain cytokines. Of eight members of SOCS family, especially SOCS1 and SOCS3 have pivotal roles in inflammation, and they inhibit signals by IFNγ and IL-6 (reviewed in Alexander and Hilton, 2004 and Lim and Cao, 2006; Shuai and Liu, 2003). PIAS proteins are constitutively expressed. PIAS family consists of seven proteins, which have a certain degree of specificity toward STATs (for example, PIAS1 and PIASy for STAT1). PIAS regulate the transcriptional activities of STATs in the nucleus, where they interact directly with phosphorylated STAT in a ligand-dependent manner and prevent the DNA binding of STATs Although, it is reported that the PIAS inhibit the activity of STATs also without affecting DNA binding (reviewed in Lim and Cao, 2006 and Wormald and Hilton, 2004).

2.6 PKR

Double-stranded RNA-dependent protein kinase (PKR) is an IFN inducible multifunctional protein, which is constitutively expressed in mammalian cells. It is also one of the four mammalian serine-threonine kinases, which phosphorylates translation initiation factor eIF2α (eukaryotic initiation factor 2 α) in response to stress signals, mainly caused by viral infection. PKR is 551 amino acids long. Its serine/threonine kinase domain is located at the C-terminal end and the RNA binding domain at the N-terminal end (reviewed in Dabo and Meurs, 2012). (Figure 5.) In non-stressed cells, PKR is monomeric in its latent state, but forms homodimers when it is activated upon binding to viral double stranded (ds) RNA structure via its N-terminal dsRNA binding domains. After homodimerization, PKR is rapidly autophosphorylated, which leads to a phosphorylation of eIF2α through the PKRs C-terminal catalytic kinase domain and finally to the inhibition of protein translation. Besides virus infection also other conditions of stress can lead to the activation of PKR, independently of dsRNA. Furthermore, PKR can be switch on by activators such as growth factors, cytokines, pro-inflammatory stimuli and oxidative stress (reviewed in García et al., 2006).

Figure 5. Structure of PKR. Simplified structure of PKR. PKR has two double stranded RNA binding domains (dsRNA) in the N-terminal end. Protein kinase domain and catalytic site are located in the C-terminal end of PKR.

In addition to PKRs ability to control protein translation, it takes part in various other cellular processes including cell growth, tumor suppression, proliferation, DNA repair and apoptosis. PKR is also involved in the regulation of many signaling pathways and a number of transcription factors (reviewed in Dabo and Meurs, 2012 and García et al., 2007). For example, the induction of cytokine expression in response to virus infection is regulated by PKR through NF-κB, IFNs and dsRNA signaling pathways by modulating certain transcription factors (Cabanski et al., 2008 and García et al., 2006). In fact, PKR is shown to phosphorylate or influence the phosphorylation of p53, STAT1 and STAT3, inhibitor κB kinase β (IKK-β), inhibitor κB β (IκB-β), the subunit of protein phosphatase 2A (PP2A), and RNA helicase at least (reviewed in García et al., 2006; Sadler et al., 2009). PKR can influence signaling pathways also through the phosphatidylinositol-3 kinase/Protein Kinase B (PI3K/AKT) pathway and transcription factors CCAAT-enhancer-binding proteins (C/EBP)α, C/EBPβ and Activating transcription factor 3 (ATF3) (reviewed in García et al., 2006).

PKR is known to activate signaling cascades involved in stress activated protein kinases. It mediates the activation of JNK and p38 MAPK in response to certain specific stimuli. Moreover, the full activation of both p38 MAPK and JNK in response to LPS or cytokines, such as IFN-γ and TNF, is dependent on PKR (Goh et al., 2000). A number of studies have addressed the involvement of PKR in the ER stress as well (Lee et al., 2007; Shimazawa et al., 2007).

2.6.1 Interaction between PKR and STAT1

Several studies have reported the interaction between PKR and STAT1 (Hsu et al., 2004; Kårehed et al., 2007; Lee et al., 2005; Ramana et al., 2000; Wang et al., 2006; Wong et al., 1997; Yoshida et al., 2009). In 1997, Wong et al., reported that PKR is able to modulate the function of STAT1, and that there is a physical association between STAT1 and PKR. They discovered that it was not a kinase-substrate interaction, since PKR failed to phosphorylate STAT1 in *in vitro* or *in vivo*. The interaction was not dependent on PKRs enzymatic activity either, even though the dsRNA binding domain of PKR

was needed for the interaction. Instead, the interaction between PKR and STAT1 was diminished upon stimulation with IFNs or dsRNA, factors which are known to induce the phosphorylation and activation of STAT1. (Figure 6a.) In addition to decreased PKR–STAT1 interaction, DNA binding of STAT1 was increased in response to IFNs or dsRNA. Hence, it is possible that other proteins or some structural changes in signaling cascades induced by IFNs or dsRNA are involved in complex dissociation (Wong et al., 1997).

Few years later the same group studied the physical PKR-STAT1 interaction further by mapping the sites where the interaction takes place; PKR amino acids 367-415, an area located within the large lobe of the kinase domain of PKR, and STAT1 amino acids 343-348, located on the back side of the DNA binding domain of STAT1. They also discovered that STAT1 functions as an inhibitor of PKR *in vitro* and *in vivo*, since the phosphorylation of eIF2alfa was enhanced in STAT1-/- cells compared with STAT1+/+ cell *in vivo,* and that correlated with the higher activation capacity of PKR in STAT-/-cells. This indicates that STAT1 has a dual role in regulating gene expression by functioning as a transcription factor and possibly as a translational regulator through PKR activation and eIF2alfa phosphorylation, while PKR activity is tightly regulated by its interaction with STAT1. (Figure 6a.) In conclusion, it appears that STAT1 provides a first line of defense against virus infection, whereas the role of PKR in viral resistance would be secondary (Wong et al., 2001). This hypothesis is supported by the fact that STAT1-/- mice are shown to be extremely sensitive to a variety of pathogens (Durbin et al., 1996; Meraz et al., 1996), whereas PKR-/- mice show only a modest sensitivity to pathogens (Abraham et al., 1999; Yang et al., 1995).

There are also several reports describing PKR and STAT1 to interact with each other by other than physical manner. Lee et al., 2005 demonstrated that in rat brain glial cells PKR is closely involved in the activation of STAT1, although it is not a direct substrate of PKR. They suggested that PKR may activate STAT1 mediated inflammatory signaling through NF-κB activation and consequent induction of IFN-β transcription. They discovered that the phosphorylation of STAT1 and the nuclear factors binding activity to GAS/ISRE elements were suppressed when PKR activity was inhibited. According to their model, LPS induced phosphorylation of PKR leads to the induction of IFN-β secretion through direct activation of NF-κB. Then, the secreted IFN-β binds to interferon receptors causing the phosphorylation tyrosine 701 and serine 727 residues of STAT1 (Lee et al., 2005). (Figure 6b.) PKR-/- bone marrow derived macrophages are also shown to exhibit defective phosphorylation of STAT1 in response to LPS (Hsu et al., 2004). This seems to be dependent on autocrine production of type I IFNs, since in macrophages, which are type I IFN receptor (IFNR I)-deficient, the phosphorylation of STAT1 was not defective (Hsu et al., 2004).

PKR seems to have an essential role in inducing the phosphorylation of STAT1 serine 727 residue in response to antiproliferative signals. For example, in PKR-null fibroblast cells the IFN-γ-induced STAT1 serine 727 phosphorylation and transactivation were both detected to be defective (Ramana et al., 2000). This is further supported by the study by Kårehed et al., 2007 where they reported that the full transcriptional activation of Fc-gamma receptor 1 (FcγRI) gene by IFN-γ in U937 monocytic cells is dependent on STAT1, and more precisely on the co-ordination of two events, the PKR-mediated phosphorylation of STAT1 serine 727 and activation of the NF-κB pathway. Treatment of U937 cells with PKR inhibitor decreased STAT1 serine 727 phosphorylation. They detected a down regulation of the phosphorylation levels of tyrosine 701as well after PKR inhibition. Their data indicate that PKR is important for STAT1 activation in U937 monocytic cells mediating the IFN-γ induced phosphorylation of STAT1 serine 727 residue (Kårehed et al., 2007).

It has been reported that PKR regulates STAT1 also in a negative manner. Wang et al, 2006 showed that this regulation occurs through activation of T-cell protein tyrosine phosphatase (TC-PTP), which in turn specifically decreases the tyrosine 701 phosphorylation of STAT1. This event is facilitated, at least in part, by the inhibition of protein synthesis through the activation of PKR and eIF2alfa phosphorylation. They detected that the phosphorylation of STAT1 tyrosine 701 residue was stronger and persisted longer in PKR-/- cells compared with PKR+/+ cells. Moreover, STAT1 tyrosine 701 phosphorylation was decreased in the presence of active PKR. So, the catalytic activity of PKR appears to exhibit its negative effect on STAT1 tyrosine 701 phosphorylation, presumably by the dephosphorylation rather than preventing the phosphorylation of STAT1. (Figure 6c.) Based on these results Wang et al., 2006 suggested that the activation of PKR influences the dephosphorylation of STAT1 and these changes in the phosphorylation status and nuclear localization of STAT1 may be reflected by changes in its transcriptional activity (Wang et al., 2006).

In 2009, Yoshida et al. demonstrated that PKR is responsible for the degradation of STAT1. In the dominant-negative PKR mutant cells, the amount of STAT1 mRNA was found to be 2.5 times higher than in the wild type cells. In addition, a significant increase in the amount of STAT1 protein was observed in these cells. Furthermore, the lack of PKR activity in dominant-negative PKR mutant cells inhibited the ubiquitination of STAT1, and ubiquitin enzyme ligand SLIM efficiently regulated the STAT1 degradation in osteoblastic cells. (Figure 6d.) Yoshida et al., 2009 also reported that high STAT1 levels stimulate the expression of IL-6 mRNA in dominant-negative PKR mutant cells, suggesting that PKR may co-operate with IL-6 and STAT1 pathways to control osteoblast differentiation during immune diseases or inflammation (Yoshida et al., 2009).

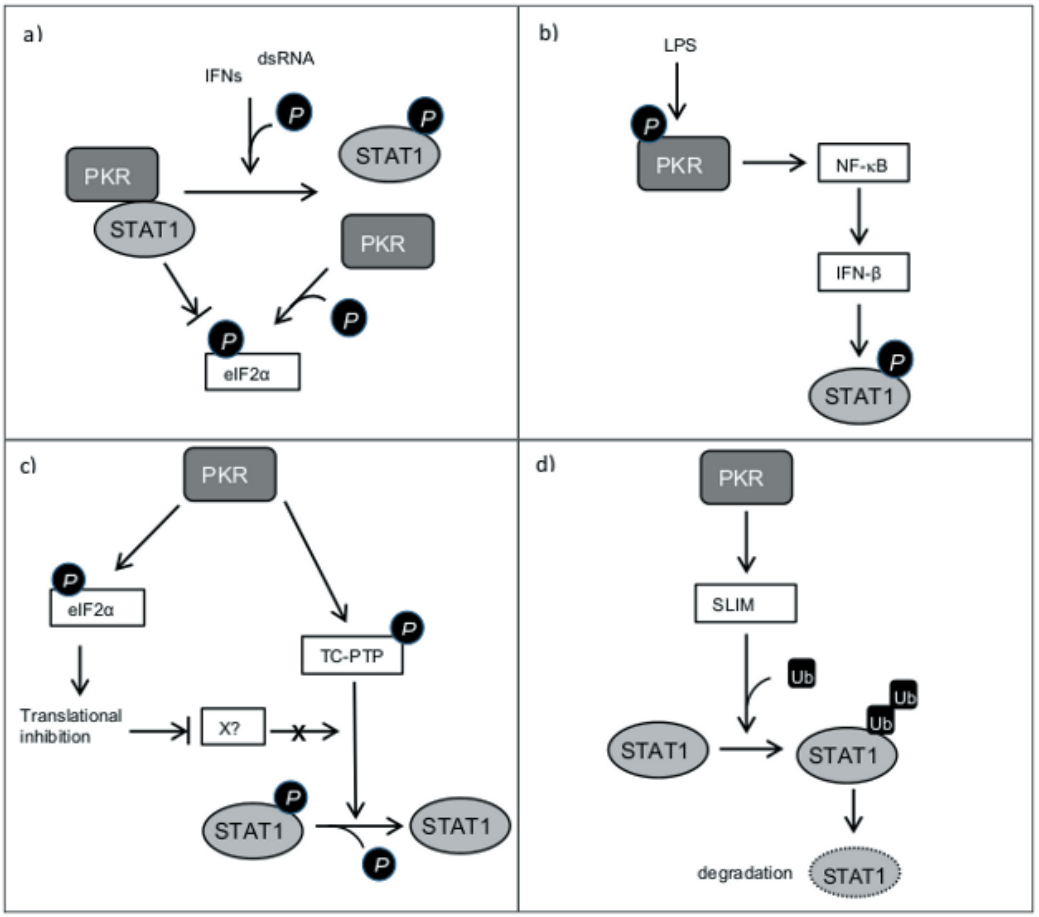

Figure 6. Interaction between PKR and STAT1. Simplified models of different PKR-STAT1 interactions. a) A physical association between STAT1 and PKR. b) STAT1 activation by PKR through NF-κB and subsequent IFN-β induction. c) Dephosphorylation of STAT1 by PKR through TC-PTP. d) Degradation of STAT1 by PKR activated SLIM. (Modified from (Lee et al., 2005; Wang et al., 2006; Wong et al., 1997; Yoshida et al., 2009)

2.7 HuR

Human antigen R (HuR, also known as ELAV1) is ubiquitously expressed RNA binding protein (RBP), which belongs to a family of embryonic lethal abnormal vision (ELAV)-like/Hu-proteins (Ma et al., 1996). It is a posttranscriptional gene regulator, which stabilizes mRNA and regulates translation by binding to AU-rich elements (ARE) of its target transcripts (reviewed in (Brennan and Steitz, 2001). Normally mRNA containing AREs are short-lived, but in conditions like cellular stress, the stabilization of ARE-containing mRNAs might be necessary so that the factors needed during stress responses can be

produced (von Roretz et al., 2011). HuR has implicated to participate in various biological processes like cell survival, immune response, inflammation, carcinogenesis, regulation of cell cycle and cell differentiation (reviewed in Brennan and Steitz, 2001). For example, HuR regulates an important inflammatory cytokine, TNF, by binding to TNFs ARE containing mRNA region and stabilize it (Dean et al., 2001). As being a major regulator of TNF mRNA HuR plays an important part in inflammatory diseases. However, in addition to promoting inflammation through regulating TNF, HuR has anti-inflammatory properties as well. HuR overexpression is shown to reduce the production of key inflammatory mediators in mice in LPS-induced model of septic shock (Katsanou et al., 2005).

HuR is predominantly located in the nucleus, but it can shuttle between the nucleus and the cytoplasm. In the cytoplasm, under severe stress, HuR can be cleaved into two cleavage products, HuR-CP1 and HuR-CP2. These cleavage products are associated with the promotion of apoptosis (Mazroui et al., 2008). The cleavage of HuR is shown to be dependent on PKR, where unphosphorylated PKR triggers HuR cleaved through FADD/caspase-8/caspase-3 pathway (von Roretz and Gallouzi, 2010).

The nuclear-cytoplasmic shuttling of HuR is linked to many signaling pathways, including MAPK and its downstream kinases. They increase HuRs cytoplasmic localization and so participate in stabilization and/or translation of TNF, IL-6, IL-8 and macrophage colony-stimulating factor (M-CSF) for example (reviewed in Doller et al., 2008).

The activation of p38 MAPK is shown directly to cause cytoplasmic accumulation of HuR. It has been suggested that the LPS induced phosphorylation of p38 MAPK leads to physical association with HuR resulting cytoplasmic accumulation of HuR (Lin et al., 2011). It has been reported that p38 MAPK also phosphorylates HuR, which results in HuRs cytoplasmic accumulation (Lafarga et al., 2009). Interestingly, HuR is also known to stabilize and facilitate the translation of MAPK phosphatase 1, MKP-1, which dephosphorylates and deactivates JNKs and p38 MAPK. Hence, it has been suggested that HuR has an essential role in the mammalian stress responses. Stress signals activate MAPKs, which leads to activation of transcription factors and activation of stress response genes, while MAPKs activated HuR stabilizes and enhances translation of stress-response mRNAs, including MKP-1. MKP-1 production then eventually leads to the dephoshorylation and deactivation of MAPKs, creating a negative feedback loop (reviewed in Kuwano and Gorospe, 2008).

2.8 Cytokines in inflammatory diseases and SpAs

Cytokines are small soluble hormone-like polypeptides or glycoproteins. They function as short-range mediators of intercellular signaling and are essentially involved in all

biological processes. They are especially important in the immune system. Cytokines can be divided into interleukins, interferons, chemokines, lymphokines, hematopoietic growth factors and tumor necrosis factor. Cytokines involved in immune responses can further be classified to pro-inflammatory or anti-inflammatory cytokines. Almost all cells secrete cytokines. However, a particular cell type can secrete several cytokines but different cell types can also secrete the same cytokine. In spite of the extreme complexity of the cytokine network, the secretion of cytokines is tightly regulated (reviewed in Dinarello, 2007 and Feldmann, 2008).

Disturbances in the cytokine network may cause a variety of different diseases. Therefore, the secretion of cytokines is used to characterize different inflammatory diseases; cytokines can be used as biomarkers to diagnose and monitor diseases. For example, cytokines such as TNF, IL-10 and IL-6 are proven to be important in SpAs and other inflammatory arthritic diseases (Claudepierre et al., 1997; rivewed in Rincon, 2012 and Waters et al., 2013). Proinflammatory cytokine TNF, together with IL-1, is considered as a master cytokine in the development of arthritis in animals, and transgenic mice overexpressing TNF spontaneously develop erosive arthritis (Keffer et al., 1991). Anti-TNF therapy has been used for years to treat inflammatory diseases, including SpAs (reviewed in Waters et al., 2013). Also, a defective production of anti-inflammatory cytokine IL-10 is linked to various autoimmune and inflammatory diseases. IL-10 knockout mice are shown to develop spontaneous IBD, and they have increased susceptibility to collagen induced arthritis (CIA), which is an animal model of RA (Asseman et al., 1999; Johansson AC et al., 2001). Furthermore, elevated IL-10 levels are found in patients with active SpA (Claudepierre et al., 1997). In addition, dysregulation in the production of IL-6, which functions both as a pro-, and anti-inflammatory cytokine, has been linked to several autoimmune diseases (reviewed in Rincon, 2012). Elevated IL-6 serum levels are measured for example in patients with RA and SpAs (Bal et al., 2007; Elkayam et al., 2000; Houssiau et al., 1988; reviewed in Song and Poddubnyy, 2011). IL-6 signaling blockade can be used to treat inflammation for example in RA, but it has proven to be effective treatment in some SpA cases as well (reviewed in Ouyang et al., 2008 and Tanaka and Kishimoto, 2012).

In addition to cytokines mentioned above, the Th17/IL-23 axis has recently become more significant in SpAs and other inflammatory diseases. Several studies have documented dysregulation of the IL-23/IL-17 axis in patients suffering from SpA (reviewed in Smith and Colbert, 2013). For example, AS patients have increased number of Th17 cells and greater levels of IL-17 (Shen et al., 2009), and they have elevated IL-23 serum levels as well (Mei et al., 2011). Moreover, misfolding of HLA-B27 molecule and occurrence of UPR have been associated with enhanced IL-23 production in transgenic rat model (DeLay et al., 2009).

IL-6, TNF and IL-10 and their roles in SpAs and in inflammation will be discussed in more detail below.

2.8.1 IL-6

IL-6 was first identified in the mid-1980s (Hirano et al., 1986; Zilberstein et al., 1986). It is a small 21 kD multifunctional cytokine, which is known to regulate immune response, hematopoiesis and function as a pro-inflammatory cytokine. IL-6 is one of the most important cytokines produced in the acute phase of inflammation, but it has an important role in chronic inflammation as well. IL-6 is produced by innate immune cells like macrophages and monocytes, B cells and some T helper cells in response to wide variety of stimuli, which represents tissue damage and stress. Its production is mainly regulated by transcriptionfactors NF-κB and C/EBPα, and changes in their gene expression (reviewed in Rincon, 2012). Furthermore, IL-6 mRNA is stabilized by HuR (Shi et al., 2012; Zhou et al., 2007).

In the early phase of infection, IL-6 production is induced immediately after Toll-like receptor (TLRs) stimulation by distinct pathogen–associated molecular patterns (PAMPS). After IL-6 is released, it binds to IL-6 receptor to initiate signal transduction. There are two kinds of IL-6 receptors; a membrane bound IL-6R and a soluble IL-6R. After IL-6 binding to the receptor, the IL-6/IL-6R complex associates with glycoprotein gp130. The complex association with gp130 initiates the activation of JAK/STAT3 pathway (reviewed in Rincon, 2012). In addition to the classic JAK/STAT3 pathway, IL-6 is shown to regulate the transcription factor C/EBPβ. Akira and Kishimoto, 1992 showed that IL-6 is able to induce both the expression and phosphorylation of C/EBPβ (Akira and Kishimoto, 1992; reviewed in Rincon, 2012)

It has been shown that PKR participates in IL-6 production. Goh and Williams, 2000 showed that the LPS induction of IL-6 mRNA, and the production of IL-6 after LPS challenge were defective in PKR-null fibroblasts (Goh et al., 2000). Also the inhibition of PKR phosphorylation is shown to severely impair the IL-6 production upon LPS challenge in alveolar macrophages (Cabanski et al., 2008). Furthermore, *mycobacteria*-induced IL-6 production was reduced as a result of PKR inhibition (Cheung et al., 2005), whereas the expression of the dominant negative PKR mutant is shown to abolish the *Herpes Simplex* virus induced IL-6 induction (Paludan, 2001).

Constant dysregulation of IL-6 production is associated with the development of many autoimmune diseases and chronic inflammatory diseases, but also with cancers (reviewed in (Tanaka and Kishimoto, 2012). Animal studies with IL-6-deficient mice revealed that these mice are resistant to the development of CIA (Alonzi et al., 1998), or they have delayed onset and less severe disease (Sasai et al., 1999). In addition, elevated

IL-6 concentrations are linked to several inflammatory diseases such as RA (Houssiau et al., 1988), Crohn´s disease (Yoshizaki et al., 1989) and AS (Gratacós et al., 1994). Elevated IL-6 concentrations are also measured in synovial fluids from patients with ReA and uSpA (Singh et al., 2007). The amount of IL-6 is likely correlated with the severity of the disease (reviewed in Rincon, 2012 and Song and Poddubnyy, 2011). Thus, nowadays IL-6 is widely used as a biomarker for disease monitoring. Furthermore, the IL-6 signaling blockade is considered as a treatment for inflammation in diseases, which have elevated IL-6 levels. For example, commercially available Tocilizumab is a humanized anti-IL6R antibody that binds both cell surface IL-6R and soluble IL-6R to block interaction with IL-6. It is now broadly accepted as an alternative treatment for RA (reviewed in Rincon, 2012). Some studies have report Tocilizumab to be effective for treating SpAs as well (Cohen et al., 2011; Koumakis et al., 2012; reviewed in Tanaka and Kishimoto, 2012).

2.8.2 TNF and IL-10

Influence of the secretion of both, TNF and IL-10, are likely to contribute to the disease outcome in SpAs (Braun et al., 1999; Yin et al., 1997). The indication that the balance between the pro-inflammatory cytokine TNF and the anti-inflammatory cytokine IL-10 is crucial in maintaining the homeostasis in the gut and joints is supported by animal studies. In particular, the importance of homeostatic balance of TNF/IL-10 against IBD is provided with the fact that mice with defective IL-10 or IL-10 signaling overproduce TNF and develop IBD (Kühn et al., 1993; Takeda et al., 1999). This is interesting since most patients with SpA have clinical or subclinical inflammation in gut in addition to joints symptoms (van den Berg and van der Heijde, 2010).

2.8.2.1 TNF

TNF is a major proinflammatory cytokine. It is a type II transmembrane protein but it has a soluble form as well. TNF is a pleiotropic cytokine which is involved in cellular death, survival, differentiation and proliferation, and is vital for the effective cellular immune response against intracellular bacteria. In fact, TNF is essential for the host defense against bacterial, viral and parasitic infections (reviewed in Waters et al., 2013). Mostly TNF is produced by macrophages, dendritic cells and T lymphocytes, but also some other tissues such as lymphoid cells, mast cells and endothelial cells can produce TNF. LPS and other bacterial products induce the release of soluble TNF. TNF has two receptors, tumor necrosis factor receptor (TNFR) 1 and TNFR2. Both membrane and soluble TNFs signals by binding to either of these receptors although membrane TNF may preferentially interact with TNFR2. TNFR1 is constitutively expressed in most

tissues, while the expression of TNFR2 is tightly controlled and it is usually expressed in cells of the immune system (reviewed in Wajant et al., 2003 and Waters et al., 2013).

The importance of TNF as a mediator of the innate immune system is observed in TNFR and TNF-deficient animals as a weakened defense against certain intracellular pathogens. These animals are more sensitive to intracellular pathogens when they are exposed to *Mycobacterium tuberculosis* (Flynn et al., 1995) or *Listeria monocytogenesis* (Rothe et al., 1994) for example. On the other hand, in some infection models, like in the acute phase of infection by *Yersinia enterocolitica*, pathological reactions are delayed in TNF knockout mice (Zhao et al., 2000).

TNF is known to induce the activation of many signaling pathways. For example, TNF activates NF-κB; it stimulates the proteolytic degradation of I-κB by proteasomes, thereby releasing NF-κB and allowing its nuclear translocation (Cheshire and Baldwin, 1997; reviewed in Wajant et al., 2003). In addition, TNF induces the activation of JNK and JNK induced MAPK cascade. It also strongly activates the p38 MAPK signaling pathway (reviewed in (Wajant et al., 2003). Furthermore, the activation and signaling of p38 MAPK leads to production and signal transduction of TNF (Lee et al., 1994).

Since TNF has such strong proinflammatory and immunostimulatory activities, it has a significant role in mediating the development of numerous autoimmune diseases. Today, anti-TNF therapy is widely and effectively used to treat RA and other inflammatory diseases, including AS, Crohn´s disease and IBD (reviewed in Wajant et al., 2003 and Waters et al., 2013). It has been suggested that both high and low TNF production is characteristic of HLA-B27 positivity and patients with SpA. For example, Repo et al, 1988 reported higher LPS-induced release of TNF in peripheral blood monocytes of HLA-B27-positive individuals or patients with previous ReA compared with HLA-B27-negative individuals (Repo et al., 1988). Whereas, Braun et al, 1999 observed that peripheral blood mononuclear cells derived from ReA patients secreted low levels of TNF, which correlated with the HLA-B27 positivity and severity of the disease upon activation with non-specific stimulants (Braun et al., 1999).

2.8.2.2 IL-10

In contrast to proinflammatory effects of TNF, IL-10 is a significant anti-inflammatory cytokine with the ability to inhibit TNF production. IL-10 is secreted by various cell types of both the adaptive and innate immune system, but its expression is minimal in unstimulated cells. The induction of the anti-inflammatory response by IL-10 is mediated through tetramer IL-10 receptor complex (IL-10R). IL-10 binding to the IL-10R activates the JAK/STAT3 pathway (reviewed in Donnelly et al., 1999 and Saraiva and O'Garra, 2010). In addition to TNF, IL-10 inhibits the expression of a number of other LPS-

inducible genes and also IFN-γ inducible genes, including many cytokines such as IL-1, IL-12 and IL-6, MHC class II molecules and inducible nitric oxide synthase (iNOS) (reviewed in Donnelly et al., 1999). IL-10 induction involves several signaling pathways such as STAT1, ERK1/2, p38 MAPK and NF-κB (Larner et al., 1993; Lehmann et al., 1994; reviewed in Saraiva and O'Garra, 2010). Hence, it has been suggested that the ability of IL-10 to inhibit the LPS induced TNF production is mediated by multiple levels. For example, one study with ARE-deficient mice indicates that IL-10 targets TNF mRNA translation by inhibiting p38 MAPK signals (Kontoyiannis et al., 2001), whereas another study with murine macrophages suggests that IL-10 destabilize TNF mRNA through a posttranscriptional mechanism (Carballo et al., 1998). Moreover, in human peripheral blood mononuclear cells, IL-10 was observed to inhibit TNF gene transcription by inhibiting NF-κB (Wang et al., 1995). There is also a contradictory study with human macrophages that suggests that the inhibition of TNF by IL-10 does not involve p38 MAPK or NF-κB signaling (Denys et al., 2002).

Since IL-10 has a significant role in immune response, defective IL-10 production has been linked to various autoimmune and inflammatory diseases. For example, IL-10 knockout mice are shown to spontaneously develop IBD (Asseman et al., 1999). These mice are also more susceptible to CIA (Johansson AC et al., 2001; Ye et al., 2014). Indeed, IL-10 is considered as a key mediator of intestinal immune homeostasis (reviewed in Saraiva and O'Garra, 2010). Despite of the anti-inflammatory nature of IL-10, elevated IL-10 levels are found in patients with active SpA (Claudepierre et al., 1997), and high levels of IL-10 are also found in patients suffering from ReA (Yin et al., 1997). Besides, the IL-10 secretion is shown to correlate with certain SpA symptoms (Claudepierre et al., 1997). The role of IL-10 in human disease is thus not so clear. High levels may also reflect attempts to control the inflammation.

3. AIMS OF THE STUDY

HLA-B27 is strongly associated with SpAs. Despite decades intensive research, the exact mechanism, how HLA-B27 causes susceptibility to these diseases is still not known. The aim of this study was to find out whether the expression of HLA-B27 molecule alters the activation of such signaling molecules and/or signaling pathways, which are involved in inflammatory reactions.

The specific aims were:

1) To study the activation of transcription factor STAT1 by phosphorylation in HLA-B27-transfected human U937 monocytic cells.

2) To study the functional interaction between STAT1 and PKR.

3) To study the secretion of cytokines involved in the immune response, more specifically the secretion of IL-6, IL-10 and TNF, in human U937 monocytic cells expressing HLA-B27 molecule.

4. MATERIAL AND METHODS

4.1 Cell culture

4.1.1 Cell lines and transfections

The human monocytic cell line U937 was obtained from American Type Culture Collection (ATCC, Rockville, MD). The U937 cell line expresses naturally the HLA class I alleles A3, A26, B18, B51, Cw1, and Cw3 (Sundstrom and Nilsson, 1976).

U937 cell line cells were transfected with the full-length 6 kbp genomic clone of human HLA-B*2705 DNA (B27g) in the pUC19 vector (Taurog et al., 1988) or with the full-length 5,1 kbp genomic clone of human HLA-A2 in the pUC9 vector (Koller and Orr, 1985) (both kind gifts from Dr. Joel D. Taurog) by electroporation. U937 cells were suspended in Roswell Park Memorial Institute (RPMI) 1640 medium containing 1,8 mM of L-glutamin (Biological Industries, kibbutz Beit-Haemek Herennek, Israel) and 1 mM sodium pyruvate (Gibco BRL,Thermo Fisher Scientific Inc., Waltham, MA). Cells were co-transfected with the pSV2neo vector to confer resistance to Geneticin [G-418] by single pulse from Bio-Rad Gene Pulser (Bio-Rad, Hercules, CA). For mock transfection, cells were transfected with pSV2neo alone.

Complementary DNA (cDNA) of HLA-B*2705 (B27cDNA) was cloned into the Bam HI site of RSV5neo vector (Carreno et al., 1993; Long et al., 1991) and transfected by liposomal transfection. Cells transfected with RSV5neo vector alone were used as a control (Both transfectants were a kind gift from Dr. David T. Y. Yu).

Three cell lines containing mutant form of HLA-B27 HC were constructed from a genomic clone of HLA-B*2705 by site-directed mutagenesis (Altered Sites, Promega, Madison, WI) in Dr. Robert A. Colbert´s laboratory. HLA-B27.E45M has one amino acid substitution at position 45 (methionine is substituted for glutamic acid) and HLA-B27. H9F mutant has a single amino acid mutation at position 9 (fenylalanine is substituted for histidine) in the B pocket of HLA-B27 HC. HLA-B27.A2B contains six amino acid mutations in the HC (H9F, T24A, E45M, I66K, C27V and K70H). The mutants were co-transfected with pSV2neo resistant vector as well (Table 2.).

All transfectants were stable and they were selected first for G-418 resistance and then for cell surface expression of the transfected class I major histocompatibility complex molecule. The cell surface expression of transfected HLA molecules was confirmed by flow cytometry as described below.

Table2. Transfected U937 cell lines.

WT HLA-B27-expressing cell lines	mutated HLA-B27-expressing cell lines	Control cell lines
B27g	B27.E45M (non-misfolding)	pSV2neo
B27cDNA	B27.A2B (non-misfolding)	RSV5neo
	B27.H9F (misfolding)	HLA-A2

4.1.2 Maintenance of the cell lines

The cells were maintained in RPMI 1640 medium, which was supplemented with 10 % fetal bovine serum (FBS) (PAA Laboratories, Linz, Austria), 1,8mM L-glutamine (Biological Industries) and 50 µg/ml of gentamicin (Biological Industries) in humified 5 % CO_2 atmosphere at 37 °C. The cells were tested for mycoplasma using a mycoplasm detection kit (Roche diagnostics, Mannheim, Germany) every time the new batch of cells was thawed for use.

4.2 Exposure of transfected cells to LPS and bacteria

Cells were diluted to a concentration of 1.0×10^6/ml and seeded in 25 cm^2 tissue culture flasks (Greiner Bio One, Frickenhausen, Germany) or in 24 well plates (Greiner Bio One). To differentiate the cells to adherent, more macrophage-like cells, they were incubated for 24 hours with 10 ng/ml phorbol myristate acetate (PMA; Sigma-Aldrich, St Louis, MO) in supplemented RPMI 1640 medium. After the incubation the adherent cells were washed once with Hank´s Balanced salt sodium (HBSS) and overlaid with supplemented RPMI 1640 medium. For LPS stimulation, final concentration of 500 ng/ml of *S. enteritidis* LPS (Sigma-Aldrich) was added to the cell culture. In some experiments the cells were stimulate with LPS right after the PMA-stimulation, without the washing step with HBSS. The cells were incubated at 37°C in 5 % CO_2 until they were harvested at the indicated time points.

For infection the strain of *S. enteritidis* used in these studies was a stool isolate from a patient diagnosed with *Salmonella*-triggered ReA. Prior the infection, bacteria were first grown for 18 hours at 37°C in tube containing 10 ml of Luria-Bertani (LB) broth. Then, 500 µl of the bacterial culture was transferred into a tube of another tube containing 10 ml of LB broth for additional 2 hours to obtain the logarithmic phase of growth. To infect the cells with *S. enteritidis,* cells were first treated with PMA as in LPS stimulation described above. Two hours before infection, adherent cells were washed once with HBSS and the medium was changed to RPMI 1640 supplemented with 10% human AB serum (Finnish

Red Cross, Helsinki, Finland). The cells were then co-cultured with *S. enteritidis* for 1 hour at 37°C in 5 % CO_2. To remove excess bacteria, cells were washed 3 times with HBSS, and overlaid with the incubation medium, supplemented RPMI 1640 containing 50 μg/ml of gentamicin to kill the remaining extracellular bacteria. The cells were incubated at 37°C in 5 % CO_2 until they were harvested at the indicated time points.

4.3 Inhibition assay

The PKR inhibitor and a negative control for PKR inhibitor, p38 MAPK inhibitor (SB202190) and negative control for p38 MAPK inhibitor (SB202474), Raf1 kinase inhibitor I (Raf1, GW5074), Casein kinase I inhibitor V (Cas,) and Src kinase inhibitor I were all used as a final concentration of 10 μM, and they were purchased from Calbiochem, Darmstadt, Germany. Inhibitors were added to the PMA-stimulated cells 15 minutes before LPS stimulation or 1 hour after infection with *S. enteritidis* (right after the excess was washed away). Additional p38 MAPK inhibitor, BIRB 796 (10 μM, Calbiochem) was added in the cell culture 15, 30, 60 or 90 minutes before LPS stimulation.

4.4 Preparation of cell extracts.

LPS-stimulated or *S.enteritidis*-infected cell were harvested at the indicated time points and washed twice with ice-cold phosphate buffered saline (PBS). The samples were frozen immediately. For protein concentration measurement, the samples were resuspended in the lysis buffer [(20 mM Tris, pH 7.5, 150 mM NaCl, 25 % etyleneglycol, 1 mM Na_3Vo_4) or (420 mM NaCl, 25% glycerol, 0,2 mM EDTA, 1,5 mM MgCl2, 20 mM HEPES pH 7,9, 0,5 mM DTT) and 0,5 mM PMSF pH 7,4; and Complete Mini Protease inhibitor coctail tablets and phosSTOP phosphatase inhibitor tablets (1 tablet/ml, Roche Diagnostics)] Samples were incubated with the lysis buffer on ice for 30-60 minutes. After centrifugation the samples at 12,000g for 20 minutes at 4°C, the supernatants were collected as whole cell extracts containing soluble proteins. Bradford protein assay (Bio-Rad, Hercules, CA) was used to measure the protein concentration of the samples.

4.5 Western blotting

Cell extracts (10–30 μg of protein) suspended in Laemmli buffer (50 mM Tris-HCl, 2 % SDS 10 % Glyseroli 0,01 % Bromphenol Blue, 5 % β-mercaptoethanol) were subjected to 7–10 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Separated proteins were transferred to nitrocellulose filters (Protran Nitrocellulose; Schleicher & Schuell, Keene, NH) using a semidry transfer apparatus (Bio-Rad). Western blot analysis was performed using rabbit polyclonal antibodies (pAb) to STAT1 (9172; Cell Signaling Technology, Danvers, MA), phospho-STAT1 (Tyr701) (9171; Cell Signaling Technology), phospho-STAT1 (Ser727) (9177; Cell Signaling Technology), HuR (6A97) (sc-71290, Santa-Cruz Biotechnology Inc., Santa Cruz, CA). Rat monoclonal antibody (mAb) to Hsc70/HSPA8 (SPA-815; Stressgen Bioreagents, Ann Arber, MI) was used as a loading control. Horseradish peroxidase-conjugated anti-rabbit antibody (Promega, Madison, WI or DAKO, Glostrup, Denmark), anti-mouse antibody (DAKO) and anti-rat antibody (Stressgen Bioreagents) were used as a secondary antibody. Blots were developed using an enhanced chemiluminescence method (Millipore, Billerica, MA). The statistical analysis was performed using Student's two-tailed t-test or Wilcoxon test for 2 independent samples.

4.6 Immunofluorescence staining and flow cytometry

To confirm the expression of transfected HLA molecules, the cells were stained with fluorescein isothiocyanate-conjugated anti-human HLA-B27 mAb (clone FD705-9EIEI0; One Lambda, Canoga Park, CA) or with anti-human HLA-A2 mAb (BB7.2; ATCC). Subclass-matched mAb recognizing chicken T cells (3G6) was used as a negative control together with FITC conjugated anti mouse ab (Sigma-Aldrich). The cells were analyzed with FACSCalibur flow cytometry (BD Immunocytometry Systems, San Jose, CA).

For STAT1 and HuR assays, PMA-maturated and LPS-stimulated and/or inhibitor-treated cells were harvested at indicated time points and washed with PBS. After harvesting, cells were fixed with 1,5-3 % formaldehyde solution for 10 min at RT. Cell were permeabilized in 100 % ice cold methanol for 10 min or 1 h. Permeabilized cells were stored at -20 °C in 100 % methanol. The cells were stained with Alexa 647-labeled phospho-specific mAb to STAT1 tyrosine 701 (612597; Becton Dickinson Biosciences, San Jose, CA) or anti-HuR (6A97) antibody (sc-71290, Santa-Cruz Biotechnology Inc.) and FITC-conjugated secondary antibody. Samples were analyzed by FACSCalibur flow cytometry (BD Biosciences).

4.7 ELISA

TNF, IL-10 and IL-6 cytokine secretions were measured using enzyme-linked immunosorbent assay (ELISA). Cell-free culture supernatants were collected 6 h or 24

h after LPS-stimulation. Commercially available antibody pairs for TNF (Mab1 and Mab11; 551220 and 554511 respectively; Becton Dickinson Pharmingen) and IL-10 (JES3-9D7 and JES3-12G8, BD Biosciences) and ELISA kit for IL-6 (Human IL-6 CytoSet ™, Invitrogen Camarillo, CA) were used. The assays were performed using protocols provided by the manufacturers of antibodies and kits. The absorbances were measured with Multiskan EX microplate photometer (Thermo Scientific, Waltham MA) at wavelength 450 nm.

4.8 Confocal microscopy

First the round glass cover slips (13-mm) were placed onto bottom of 24-well tissue culture plate (Greiner Bio One) wells. The cells were diluted to a concentration of $1–2\times10^6$/ml and seeded into the wells. PMA-maturated cells were subjected to PKR inhibition and/or infected with *S. enteritidis* as described above. Cells were fixed with 3.7% formaldehyde 2 hours post infection and permeabilized with 100% ice cold methanol. Cells were stained with STAT1 antibody (1:50; Cell Signaling Technology) and Alexa fluor 568 secondary antibody (1:800; Invitrogen life technologies, Carlsbad, CA). The nuclei were stained with Hoechst nuclei stain. Zeiss LSM510 META laser scanning microscope (Carl Zeiss, Jena, Germany) with a 100× oil objective was used to visualize the cells.

5. RESULTS

Roman numerals (I-IV) refer to the original communications.

In this study, we used human monocytic U937 cell line cells. Cells were stably transfected with either the full-length genomic clone of human HLA-B27 DNA or with the complementary DNA of HLA-B27. For control, U937 cells were transfected with the antibiotic resistance vector pSV2neo, which was also co-transfected to genomic HLA-B27 transfectants, or with the RSV5neo vector, in which the HLA-B27 cDNA was inserted. As an additional control, we used U937 cells transfected with HLA-A2 molecule. HLA-A2 is a MHC class I molecule, which is not associated with SpAs. To study the effects of HLA-B27 HC misfolding, the cells were transfected with HLA-B27 molecules carrying a mutation or mutations in their HC. B27.E45M mutant has a single amino acid mutation in the B pocket of the HC, where methionine is substituted for glutamic acid at position 45. This mutation prevents the misfolding of HLA-B27 molecule (Dangoria et al., 2002; Mear et al., 1999). In contrast, B27.H9F in which phenylalanine is substituted for histidine at position 9 in the B pocket of the HC, misfolds even more easily than the wild type HLA-B27 (Penttinen et al., 2004b). The B27.A2B mutant carries six mutations (H9F, T24A, E45M, I66K, C67V and H70K), resembling the B pocket of HLA-A2 molecule, and does not misfold (Dangoria et al., 2002; Mear et al., 1999).

5.1 Expression of STAT1 in HLA-B27-transfected human U937 monocytic cells (I, II)

In our earlier studies, we have seen evidence that the expression of HLA-B27 molecule may alter the major signaling pathways involved in inflammatory reactions (Penttinen et al., 2002; Sahlberg et al., 2007; Sahlberg et al., 2012). For example, the expression and activity of PKR is altered in HLA-B27-expressing U937 cells (Sahlberg et al., 2012). Interestingly, PKR is known to interact and regulate the signaling molecule STAT1 (Ramana et al., 2000; Wang et al., 2006; Wong et al., 1997). STAT1 is a transcription factor, which has an important role in immune response against viruses, bacteria and parasites, and is a major component of the cellular response to IFN-γ (reviewed in Najjar and Fagard, 2010). These facts prompted us to study the expression and activation of STAT1 in HLA-B27-expressing cells. We used a western blot method. We observed a strong expression of the STAT1 in HLA-B27-expressing cells as well as in vector-

transfected control cells (mocks). However, in the HLA-B27-transfected cells the expression of STAT1 molecule was slightly stronger compared with vector-transfected control cells. The expression of STAT1 was observed already in PMA-maturated cells, even though the stimulation of the cells with LPS or infection with *S.enteritidis* did increase the expression of STAT1 slightly.

5.2 Phosphorylation of STAT1 tyrosine 701 residue in HLA-B27-transfected cells (I, II)

The activity of STAT1 is regulated by phosphorylation. The most important phosphorylation site of STAT1 is the tyrosine residue at position 701. It has been shown that the phosphorylation of this residue is required and sufficient for the STAT1´s dimerization, nuclear localization and DNA binding (Shuai et al., 1993; Wen et al., 1995). Therefore, we studied whether the expression of HLA-B27 has an effect on STAT1´s activity, especially on the phosphorylation of STAT1. First we studied the phosphorylation of STAT1 tyrosine 701 residue with the western blot method. We discovered that the phosphorylation of STAT1 tyrosine residue 701 was strongly enhanced in HLA-B27-expressing cells, whereas in vector-transfected control cell lines the phosphorylation was weak. The enhanced phosphorylation in HLA-B27-expressing cells was detected even before addition of external triggers like LPS or infection with *S.enteritidis*. Although the stimulation of the cells with LPS or infection with bacteria did enhance the phosphorylation of STAT1 tyrosine 701 in all cell lines studied, the effect was more robust in HLA-B27-expressing cells compared with vector-transfected control cells.

To study whether the tendency of HLA-B27 HCs to misfold affects the phosphorylation of STAT1 tyrosine 701, we used cells expressing the mutated form of HLA-B27, B27.E45M cells, which do not misfold. In B27.E45M cells the phosphorylation of STAT1 tyrosine 701 was weak, even after the stimulation with LPS or infection with *S.enteritidis*, as in vector-transfected control cell line. (Figure 7.)

To further confirm the results observed with the western blot method, the intensity of the STAT1 tyrosine 701 phosphorylation was measured with the flow cytometry. The intensity of STAT1 tyrosine 701 phosphorylation was statistically significantly higher in HLA-B27-expressing cell compared with vector-transfected control cells.

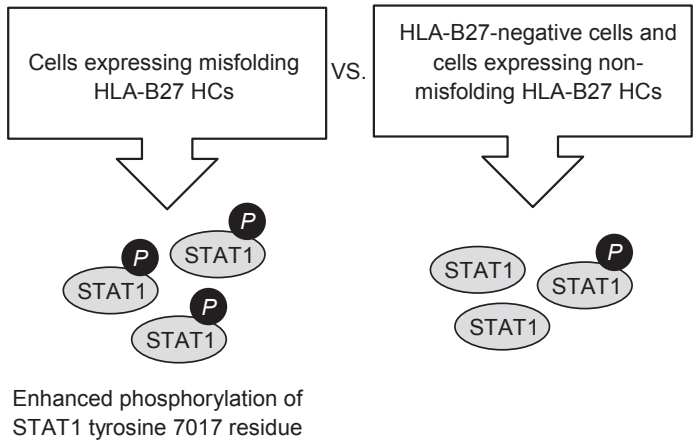

Figure 7. The phosphorylation of STAT1 tyrosine 701 residue. Phosphorylation of STAT1 tyrosine 701 residue is enhanced in cells expressing misfolding HLA-B27 HCs (WT HLA-B27) compared with HLA-B27-negative cells (vector transfected control cells) and cells expressing misfolding HLA-B27 HCs (B27.E45M).

5.3 Phosphorylation of STAT1 serine 727 residue in HLA-B27-transfected cells (I, II)

The other important phosphorylation site of STAT1 is serine 727 residue in the C-terminal end of STAT1. This residue is missing from the STAT1β isoform as a result of different splicing (Schindler et al., 1992). It has been reported that the phosphorylation of this residue is required for the maximal induction of STAT1´s activity. STAT1 lacking the serine 727 residue is able to dimerize, localize to the nucleus and bind to DNA, but for example the transcription of IFN-γ-dependent genes is only about 20 % of the maximal induction (Wen et al., 1995). Furthermore, STAT1 serine 727 residue is shown to be extremely important for bacterial clearance in mice (Varinou et al., 2003). We studied the phosphorylation of STAT1 serine 727 residue in HLA-B27-transfected U937 cells with the western blot method. A fairly strong phosphorylation of this residue was detected in all U937 transfectants already after PMA maturation. Yet, the stimulation of the cells with LPS or infection with *S. enteritidis* did enhance the phosphorylation of STAT1 serine 727 residue slightly in HLA-B27-expressing cells compared with vector-transfected control cells. However, in HLA-B27-expressing cells the phosphorylation of STAT1 serine 727 was prolonged upon *S. enteritidis* infection compared with mock cells. In HLA-B27-expressing cell the level of the STAT1 serine 727 phosphorylation stayed high for several hours, and almost at the same level as it was at its highest point after infection, while in mock cells the phosphorylation decreased faster.

The prolonged phosphorylation of STAT1 serine 727 residue seems to be dependent on the misfolding tendency of HLA-B27 HCs. The B27.H9F mutant, which is shown to misfold even more easily than the wild type HLA-B27, behaved like the wild type HLA-B27-expressing cells, and the phosphorylation of STAT1 serine 727 stayed high, even in the longer time points. In contrast, in non-misfolding B27.E45M mutant, the phosphorylation of the serine 727 residue decreased fast after the first induction with *S. enteritidis*. (Figure 8.)

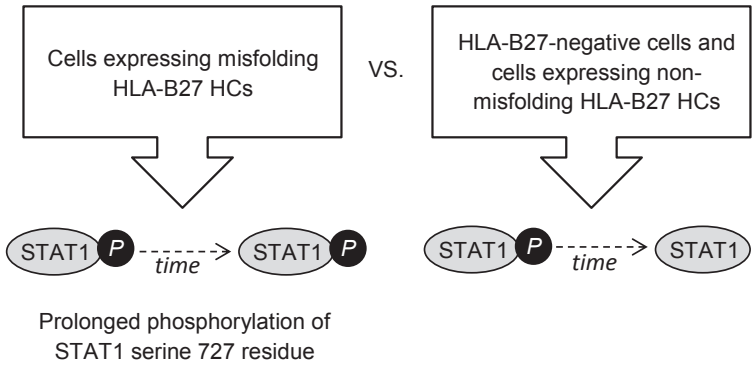

Figure 8. The phosphorylation of STAT1 serine 727 residue. In *S. enteritidis* infected cells, the phosphorylation of STAT1 serine 727 residue is prolonged in cells expressing misfolding HLA-B27 HCs (WT HLA-B27 and B27.H9F) compared with HLA-B27-negative cells (vector transfected control cells) and cells expressing misfolding HLA-B27 HCs (B27. E45M).

5.4 PKR Inhibition (I, II)

To study whether PKR modulates the phosphorylation of STAT1 we used a specific commercially available inhibitor to inhibit PKRs activity in the cells. The inhibitor prevents the autophosphorylation of PKR by blocking the ATP binding site on PKR molecule. Interestingly, we discovered that in HLA-B27-expressing cells, the phosphorylation of STAT1 tyrosine 701 residue turn out to be strongly dependent on PKR activity. The phosphorylation of STAT1 tyrosine 701 residue was almost completely inhibited in cells, which were treated with the PKR inhibitor. This was seen in LPS stimulated HLA-B27-expressing cells as well as in HLA-B27-expressing cells, which were maturated with PMA, but not stimulated with the external trigger (and in which the enhanced STAT1 tyrosine 701 phosphorylation was detected before stimulation). In vector-transfected control cells the inhibition of PKR decreased the phosphorylation of STAT1 tyrosine 701 to some extent in LPS stimulated cells but had only a minor effect on only PMA-maturated cells. (Figure 9.)

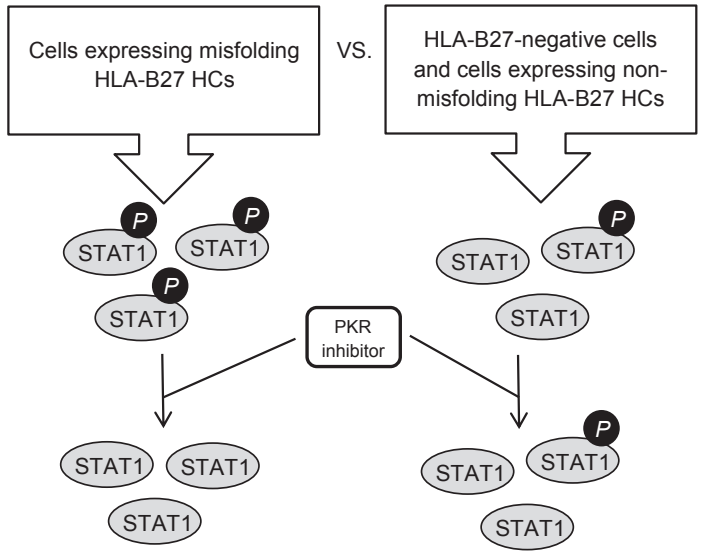

Figure 9. PKR regulates the phosphorylation of STAT1 tyrosine 701. In cells expressing misfolding HLA-B27 HCs (WT HLA-B27) the use of PKR inhibitor decreased the phosphorylation of STAT1 tyrosine 701 residue, but not in HLA-B27-negative cells (vector transfected control cells).

Because of the dramatic effect of PKR inhibition on the STAT1 tyrosine 701 phosphorylation, we studied how the inhibition of PKR affects the phosphorylation of STAT1 serine 727 residue. In contrast to STAT1 tyrosine 701 site, the inhibition of PKR had no effect on serine 727 phosphorylation in LPS stimulated HLA-B27-expressing cells, but it did inhibit the STAT1 serine 727 phosphorylation in only PMA-maturated HLA-B27-expressing cells. On the other hand, in vector-transfected control cells the phosphorylation of STAT1 serine 727 was effectively decreased in both LPS stimulated cells and PMA-maturated cells as a result of PKR inhibition.

Since we detected that the phosphorylation of STAT1 serine 727 residue was prolonged in HLA-B27-expressing cells upon *S.enteritidis* infection, we studied the interaction between STAT1 and PKR in more detail. We used misfolding B27.H9F mutant cells and non-misfolding B27.E45M mutant cells. Supporting our earlier results, in cells expressing wild type HLA-B27 or B27.H9F, and in which the phosphorylation of STAT1 serine 727 was prolonged, the inhibition of PKR had only a minor effect on the serine 727 phosphorylation at early time points (2h after infection). Whereas in vector-transfected control cells and in non-misfolding B27.E45 mutated cells, the inhibition of PKR did diminish the phosphorylation of STAT1 serine 727 effectively. Interestingly, at later time points the phosphorylation of STAT1 serine 727 was dependent on PKR in all cells studied. (Figure 10.)

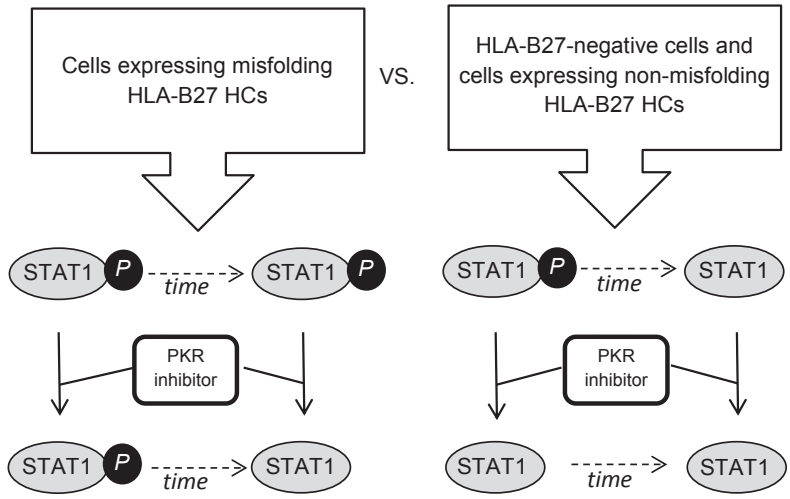

Figure 10. PKR regulates the phosphorylation of STAT1 serine 727. In HLA-B27-negative cells (vector transfected control cells) and in cells expressing non-misfolding HLA-B27 HCs (B27.E45M) the use of PKR inhibitor decreased the phosphorylation of STAT1 serine 727 effectively already at early time points (2 hours after infection with S. enteritidis), but not in cells expressing misfolding HLA-B27 HCs (WT HLA-B27 and H9F). In later time points the phosphorylation of STAT1 serine 727 was dependent on PKR in all cell lines.

Because the phosphorylation of STAT1 serine 727 residue is associated with the p38 MAPK pathway (Kovarik et al., 1999), and we have earlier seen evidence that the p38 MAPK pathway is dysregulated in HLA-B27-expressing U937 cells (Sahlberg et al., 2007), we studied whether the inhibition of p38 MAPK activity affects the phosphorylation of STAT1 serine 727 in our cell line model. The p38 inhibitor, SB202190, inhibits the phosphorylation of p38 MAPK, which is needed for its kinase activity. In contrast to our expectations, the inhibition of p38 MAPK had no effect on STAT1 serine 727 phosphorylation in HLA-B27-expressing cells or in vector-transfected control cells in this experiment. The inhibition of p38 MAPK activity did not have an influence on STAT1 tyrosine 701 phosphorylation in HLA-B27-expressing cells either (data not shown).

5.5 Localization of STAT1 (II)

As a transcription factor, STAT1 needs to be transported to the nucleus so that it can bind to DNA (reviewed in Ivashkiv and Hu, 2004). Since we detected that the phosphorylation of STAT1 tyrosine 701 residue, which is needed for the nuclear localization, is enhanced in cells expressing misfolding HLA-B27 molecules, and that the phosphorylation of serine 727 residue is prolonged in these cells ensuring the maximal transcriptional

activity, we studied the localization of STAT1 with confocal microscopy. Localization studies revealed that there was approximately 40% more STAT1 localized in the nucleus of HLA-B27-expressing cells, even before the addition of external triggers (LPS or *S. enteritidis*), than in mock cells.

Since the phosphorylation of STAT1 was found to be dependent on PKR on both tyrosine 701 and serine 727 residues, we studied whether the inhibition of PKR has an effect on STAT1 localization. In mock cells the inhibition of PKR did prevent the nuclear localization of STAT1 about 17 %. In contrast, in the HLA-B27-transfected cells the inhibition of PKR did not seem to have any effect on STAT1 localization.

5.6 Expression of HuR in HLA-B27-transfected cells (III)

HuR is known to participate in the regulation of some signaling pathways involved in the inflammatory response and cell stress (reviewed in Doller et al., 2008 and Kuwano and Gorospe, 2008). In addition, we have seen evidence that the expression of HLA-B27 molecule may alter the signaling pathways involved in inflammatory reactions. For example, the behavior of PKR and p38 MAPK, which are both also known to participate in the regulation of HuR (Lafarga et al., 2009; Lin et al., 2011; von Roretz and Gallouzi, 2010) are altered in HLA-B27-expressing cells (Penttinen et al., 2002; Sahlberg et al., 2007; Sahlberg et al., 2012). That intrigued us to study the expression of HuR in HLA-B27-expressing cells. First, the expression of HuR was studied with flow cytometry. The expression of HuR was detected to be similar in all cell lines studied. However, results obtained with western blot revealed that the cells expressing the misfolding forms of HLA-B27 (wild type HLA-B27 or B27.H9F) expressed more full-length HuR than vector-transfected control cells and non-misfolding B27.E45M mutant cells. This was seen already before LPS stimulation or infection with *S. enteritidis*. The stimulation or infection itself did not increase HuR expression in these cells. Furthermore, wild type HLA-B27-expressing cells and B27.H9F-expressing mutated cells generated notably less HuRs cleavage products, especially HuR-CP2, than mock and non-misfolding B27.E45M-expressing cells.

The same PKR inhibitor we used in STAT1 studies was used to study the role of PKR in HuR expression. The inhibition of PKR activity increased the expression of full-length HuR in LPS stimulated mock and non-misfolding B27.E45M-expressing cells, but also in misfolding B27.H9F cells. In *S. enteritidis*-infected cell the inhibition of PKR increased full-length HuR expression in mock and B27.E45M-expressing cell, but had only a minor effect on cells expressing misfolding HLA-B27. The generation of HuR-CPs however, was increased in all cell lines as a result of PKR inhibition.

The inhibition of p38 MAPK with the SB202190 inhibitor decreased the expression of full-length HuR in both LPS-stimulated and *S. enteritidis* infected mock and non-misfolding B27.E45M-expressing cells, while the effect was more profound in *S. enteritidis* infected cells. In cells, which express the misfolding forms of HLA-B27 molecules, the inhibition of p38 MAPK had no effect on HuR expression. Furthermore, another p38 inhibitor, BIRB, was used to study the involvement of p38 MAPK in HuR expression in LPS stimulated cells. The full-length HuR expression level was decrease after inhibition with BIRB in mock cells. A minor decrease was also detected in B27.E45M-expressing cells but BIRB had no effect on full-length HuR expression in HLA-B27 and B27.H9F-expressing cells. However, the amount of HuR-CP1 was increased in HLA-B27-expressing cells as a result of BIRB, whereas in mock cells the level of HuR-CP2 was increased.

Besides p38 MAPK, the SB202190 inhibitor is known to affect the kinase activities of Src, casein 1 and Raf1 kinases. Hence, the inhibitors of these kinases were used to study the expression of HuR. However, they had no effect on HuR expression in any cell lines studied, although Raf1 inhibition did decreased the expression of full-length HuR and HuR-CP1 in HLA-B27-expressing cells a little.

5.7 Secretion of cytokines in HLA-B27-transfected cells (III, IV)

Cytokines are involved in the pathogenesis of many infectious and inflammatory diseases and their secretion is tightly regulated. Thus, the proper coordination of pro-inflammatory and anti-inflammatory cytokines is essential for cells to defend against infections triggered by different pathogens (reviewed in Fresno et al., 1997). To study the secretion of cytokines in U937 monocytic cells, and possible differences in cytokine production between the HLA-B27-transfected cells and control cells, we used an enzyme-linked immunosorbent assay (ELISA).

IL-6 is a pleiotropic cytokine, which has a key role in the acute phase of inflammation but in chronic inflammation it acts rather in pro-inflammatory manner (reviewed in Gabay, 2006). Furthermore, its role in the clearance of infections with intracellular bacteria and viruses has been reported (Kopf et al., 1994). We discovered that the secretion of IL-6 was strongly elevated in HLA-B27-expressing cells compared with control cells. The PMA-maturated cells did not secrete a measurable amount of IL-6 in any cell line studied, but the LPS stimulation induced IL-6 secretion in HLA-B27-expressing cells already 6 hours after LPS stimulation, whereas in vector-transfected control cells and in HLA-A2-transfected cells the IL-6 secretion was undetectable. 24 hours after LPS stimulation the IL-6 secretion was induced in all cell lines. However, in

HLA-B27-expressing wild type cells the secretion of IL-6 was approximately 10 times higher compared with control cell lines. Furthermore, the high IL-6 secretion seems to be correlated with the misfolding of HLA-B27 HC, since the enhanced IL-6 secretion was observed also in misfolding B27.H9F mutant-transfected cells. In fact, the secretion of IL-6 in B27.H9F transfectants was over 3 times higher 6 hours after LPS stimulation and almost 2 times higher 24 hours after LPS stimulation compared with the wild type HLA-B27-expressing cells. In contrast, the B27.A2B mutant, which contains six single amino acid mutations in its HC, and does not misfold, behaved like vector-transfected and HLA-A2-transfected control cell lines. (Figure 11.)

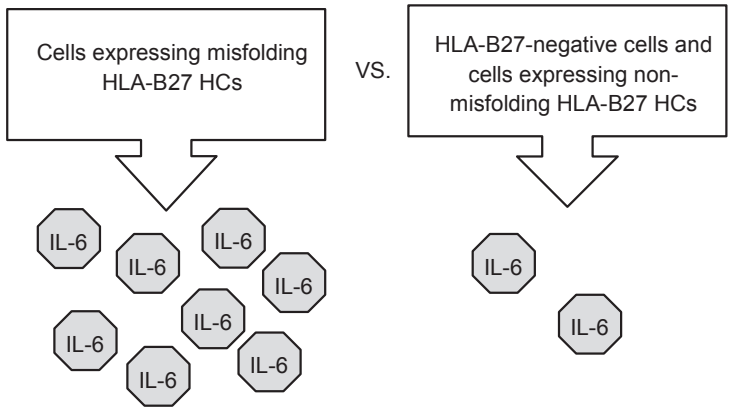

Figure 11. The secretion of IL-6. The secretion of IL-6 is elevated in LPS stimulated cells expressing misfolding HLA-B27 HCs (WT HLA-B27 and B27.H9F) compared with HLA-B27-negative cells (vector transfected control cells and HLA-A2) and in cells expressing non-misfolding HLA-B27 HCs (B27.A2B).

PKR is shown to influence the IL-6 secretion in LPS challenged alveolar macrophages (Cabanski et al., 2008). In addition, we detected earlier that the expression and activation of PKR are altered in HLA-B27-expressing cells (Sahlberg et al., 2012). Hence, to study whether PKR influences the IL-6 secretion in HLA-B27-transfected cells, we used the same commercially available PKR inhibitor as earlier. Interestingly, the inhibition of PKR abolished the IL-6 secretion completely in all cell lines we studied, including the misfolding and non-misfolding forms of the HLA-B27-transfectants as well as the control cell lines. This suggests that the IL-6 secretion in transfected U937 monocytes is dependent on PKR. The PKR inhibitor control molecule did not have a significant effect on IL-6 secretion.

The balance between pro-and anti-inflammatory cytokines, TNF and IL-10, is essential for the proper regulation of inflammation (Takeda et al., 1999; Yin et al., 1997). It has been shown earlier that the secretions of TNF and IL-10 are elevated in HLA-B27-

expressing cells (Ekman et al., 2002). Furthermore, it is known to that PKR and p38 MAPK are involved in TNF and IL-10 production (Cabanski et al., 2008; Campbell et al., 2004; Dobreva et al., 2009; Kontoyiannis et al., 2001; Lin et al., 2010). However, in this study the overall level of the LPS induced TNF secretion was similar in the HLA-B27-transfected wild type cells, B27.H9F mutant cells and in vector-transfected control cells. Nevertheless, the secretion of TNF was found to be totally dependent on PKR. The inhibition of PKR prevented the TNF secretion in all U937 transfectants studied. Similar to the TNF, no major differences were detected in IL-10 secretion between the cell lines before or after LPS stimulation. The inhibition of PKR also diminished the IL-10 secretion very effectively, almost to zero, in all cell lines studied.

Since p38 MAPK is known to participate in the regulation of TNF and IL-10 signaling (Campbell et al., 2004; Dobreva et al., 2009), we studied how inhibition of the p38 MAPK affects the secretion of these cytokines in U937 cells. The inhibition of p38 MAPK did not have a notable effect on TNF secretion in any cell line. However, the secretion of IL-10 was observed to be strongly dependent on p38 MAPK activity; the inhibition of p38 MAPK activity prevented the IL-10 secretion very effectively in all cell lines studied.

6. DISCUSSION

HLA-B27 is a well-known risk factor for developing certain types of rheumatic diseases, SpAs (Brewerton et al., 1973; Schlosstein et al., 1973). For example, nearly 80 % of the patients suffering from ReA are HLA-B27-positive (reviewed in Hannu, 2011). The interaction between HLA-B27 molecule and the susceptibility to these diseases has been studied intensively for decades.

Our research group has studied HLA-B27 and its role in host cells encountering ReA-triggering bacteria for many years. For example, it was discovered that the elimination of *S. enteritidis* was impaired in the HLA-B27-transfected cells compared with control cells (Laitio et al., 1997; Virtala et al., 1997). In addition, HLA-B27-expressing cells were more permissive for the intracellular replication of *S. enteritidis* (Penttinen et al., 2004b). Interestingly, this phenotype appears to be dependent on HLA-B27 misfolding, since in cells expressing mutated forms of HLA-B27 HC, and which do not misfold anymore, the replication of intracellular *S. enteritidis* was not detected (Penttinen et al., 2004b). Our more recent studies show evidence that some signaling pathways involved in inflammation may be altered in HLA-B27-expressing cells. These studies indicate that the p38 MAPK signaling pathway has a significant role in regulating the replication of *S. enteritidis* in U937 cells, and that in cells expressing misfolding HLA-B27, this signaling pathway does not function properly (Sahlberg et al., 2007). In addition, the signaling molecule PKR was discovered to be overexpressed and hypophosphorylated in cells expressing misfolding HLA-B27 molecule (Sahlberg et al., 2012). These findings prompted us to study in more detail about how the expression of HLA-B27 molecule affects signaling pathways, and hence could contribute to the disease susceptibility.

6.1 STAT1 in IFN signaling

STAT1 is a transcription factor, which has an important role in regulating the inflammatory response in cells. Furthermore, it is a major mediator of IFN-γ-dependent responses. One way to regulate the activity of STAT1 is phosphorylation. STAT1 has two major phosphorylation sites, tyrosine 701 and serine 727 (reviewed in Najjar and Fagard, 2010). Our results show that in HLA-B27-expressing cells the tyrosine residue 701 of STAT1 is constitutively phosphorylated, suggesting that the expression of HLA-B27 molecule can spontaneously induce the activation of STAT1, even in the absence

of external triggers like LPS or bacterial infection. Since it has been shown that the phosphorylation of STAT1 tyrosine 701 residue is required and sufficient for STAT1 dimerization, nuclear localization and DNA binding, and to induce IFN-dependent gene transcription (Shuai et al., 1993; Wen et al., 1995), the constitutive phosphorylation of STAT tyrosine 701 residue may lead to an overexpression of IFN-dependent genes, as well other STAT1-dependent genes. This assumption is supported by the transgenic animal study, where macrophages isolated from HLA-B27 transgenic rats overexpress various IFN-regulated genes when they exhibit UPR activation (Turner et al., 2005). On the other hand, macrophages derived from patients suffering from AS showed evidence of reduced IFN-γ mRNA levels and they expressed less IFN-γ upregulated genes than macrophages from healthy control patients (Smith et al., 2008). A low IFN-γ production could be a kind of defense mechanism for HLA-B27-expressing cells to survive, since constant STAT1 activation by phosphorylation in HLA-B27-expressing cells could lead to an over production of IFN-γ and cell death. Incidentally, we have been unable to detect IFN-γ production in our U937 cells (unpublished observation), but cultured macrophages alone express very low levels of IFN-γ in any case (reviewed in Bogdan and Schleicher, 2006).

Our localization studies with confocal microscopy revealed that more STAT1 resides in the nucleus of HLA-B27-expressing cells than in the nucleus of the control cells, indicating that the enhanced activation of STAT1 is functionally relevant. This further supports the idea that HLA-B27 expression alone is sufficient to cause the activation of STAT1.

6.2 STAT1 and ER stress

The accumulation of misfolded HLA-B27 HCs in the ER can cause ER stress and the activation of the UPR in the cells (reviewed in Colbert et al., 2010). However, stably-transfected cells expressing HLA-B27, including the U937 cell line cells used in this study, show no signs of acute UPR activation (Penttinen et al., 2004b) (and unpublished observations). It is possible that in cells which are selected for survival while expressing misfolding proteins, other mechanism(s), beside the UPR, are involved in managing the chronic ER stress. Based on the dual role in regulating the inflammatory response, STAT1 is proposed to execute a homeostatic role in the inflammatory response (reviewed in Ivashkiv and Hu, 2004). Thus, one possible explanation for enhanced STAT1 activation in U937 cells expressing HLA-B27 molecule is that cells stably expressing HLA-B27 could be adapted to a chronic stress, with STAT1 activation functioning as a compensatory mechanism to protect these cells from dying. Indeed, ER stress is known

to induce STAT1 serine 727 phosphorylation (Kovarik et al., 1999), which we detected to be prolonged in HLA-B27-expressing U937 cells upon *S. enteritidis* infection.

Severely misfolded or aggregated proteins residing in the ER can be alternatively transported to cytosol and degraded in autophagosomes (reviewed in Colbert et al., 2014; Tsai et al., 2002). Interestingly, STAT1 is shown to promote the autophagy activity by inhibiting an autophagy regulatory molecule Ras. This regulatory mechanism depends on the phosphorylation of STAT1 tyrosine 701 residue (Wang et al., 2008). In addition to STAT1, it has been reported that the p38 MAPK and PKR signaling pathways promote the activity of autophagy (Talloczy et al., 2002; Tang et al., 2008). Since we have detected that these signaling pathways are dysregulated in HLA-B27-expressing U937 cells (Sahlberg et al., 2007; Sahlberg et al., 2012), it is possible that the enhanced phosphorylation of STAT1 tyrosine 701 is functioning as a compensatory mechanism to enhance the autophagy through Ras, and hence protect these cells from dying.

It has been reported that the differentiation process of monocytes to macrophages itself can induce UPR. However, this physiological UPR results from an increase in *de novo* protein synthesis and differs from ER stress-induced UPR activation. Although, it may actually also protect macrophages from ER stress-induced cell death and apoptosis (Dickhout et al., 2011).

6.3 STAT1 and abnormal bone formation

Abnormal bone formation is a characteristic of AS. Despite active research the mechanisms underlying this process are still not identified (reviewed in Dakwar et al., 2008). Now, our results show increased activation of STAT1 in cells expressing HLA-B27 molecule, which is a well-known risk factor for SpA, including AS. Interestingly, it has been shown that STAT1 is involved in the regulation of bone remodeling; it inhibits bone formation and fracture healing (Kim et al., 2003; Tajima et al., 2010). Both type I and type II interferons (IFN-α/β and IFN-γ) are shown to be critical for the regulation of the skeletal system by suppressing osteoclastogenesis (a process of generation of bone resorbing cells). As a major mediator of interferon signaling, STAT1 has a critical role in this process too (Takayanagi et al., 2002; Takayanagi et al., 2000). For example, STAT1$^{-/-}$ mice exhibit an increased osteoclast number and enhanced bone resorption, suggesting that IFN-STAT1 signaling is an important suppressor of osteoclastogenesis (Tajima et al., 2010; Takayanagi et al., 2002). However, in spite of enhanced osteoclastogenesis, bone mass is increased in STAT1 -/- mice, they have increased bone formation and osteoblast differentiation (Kim et al., 2003). STAT1 regulates bone formation also independently of IFN signaling. STAT1 has been shown to interact in its latent unphosphorylated form

with Runx2, which is an essential transcription factor for osteoblast differentiation, preventing nuclear translocation of Runx2 (Kim et al., 2003). Thus, the enhanced STAT1 activation observed in HLA-B27-expressing cells could be involved in abnormal bone formation, which is characteristic symptom of AS. On the other hand, HLA-B27 transgenic rats with systemic inflammation are shown to have normal bone formation compared with control animals, in spite of increased bone resorption (Papet et al., 2008).

6.4 Phosphorylation of STAT1 serine 727 residue and p38 MAPK

According to our results, the phosphorylation of STAT1 serine 727 is prolonged in HLA-B27-expressing cells upon infection with *S. enteritidis*. Since the phosphorylation of serine 727 is important for STAT1´s maximal transcriptional activity (Wen et al., 1995), the prolonged phosphorylation of STAT1 serine 727 site could induce the exaggerated inflammatory response in HLA-B27-expressing cells. In fact, it has been reported that the HLA-B27-positive individuals suffering from salmonella infections have more severe symptoms (Ekman et al., 2000). STAT1 serine 727 residue is shown to be important in bacterial clearance as well, since mice expressing mutated STAT1 with mutation in serine 727 residue, are highly sensitive to bacterial infections (Varinou et al., 2003). As mentioned above, the elimination of *S. enteritidis* is impaired in the HLA-B27-transfected cell lines cells (Laitio et al., 1997; Virtala et al., 1997), and these cells are more permissive for intracellular replication of *S. enteritidis* compared with control cells (Penttinen et al., 2004b). Thus, the prolonged activation of STAT1 serine 727 residue observed in HLA-B27-expressing cells could help these cells to manage the excess bacterial load detected in HLA-B27-expressing cells.

According to literature, the phosphorylation of STAT1 serine 727 is mediated, at least partly, by p38 MAPK (Kovarik et al., 1999). Especially stress conditions like ER stress or exposure to LPS are known to induce STAT1 serine 727 phosphorylation through the p38 MAPK pathway (Kovarik et al., 1999). Interestingly, we observed earlier that the p38 MAPK pathway is dysregulated in U937 cells expressing HLA-B27 (Sahlberg et al., 2007). However, our results indicate that the phosphorylation of STAT1 serine 727 residue is not dependent on the p38 MAPK pathway in our U937 cell line model, since the inhibition of p38 MAPK had no effect on STAT1 serine 727 phosphorylation in any U937 transfectants we studied. This might be explained by the involvement of the other factors known to participate in the phosphorylation of STAT1 serine 727 residue. For example, the IL-1-mediated phosphorylation of STAT1 serine 727 is shown to require IL-1 receptor-associated kinase (IRAK), (Nguyen et al., 2003), whereas p38 MAPK is needed for TNF mediated STAT1 serine 727 phosphorylation (Kovarik et al., 1999). This

is interesting since both TNF and IL-1 are master cytokines in regulating the development of joint inflammation in arthritis (reviewed in van den Berg, 2001). In addition, PKCδ is also shown to participate in TLR4 induced STAT1 serine 727 phosphorylation (Rhee et al., 2003).

6.5 Interaction between STAT1 and PKR

It has been shown that PKR is involved in STAT1 activation (Hsu et al., 2004; Lee et al., 2005; Ramana et al., 2000; Wang et al., 2006; Wong et al., 1997; Yoshida et al., 2009). Since we detected disturbed PKR signaling and now enhanced STAT1 activation in HLA-B27-expressing cells, we studied how PKR affects the STAT1 phosphorylation in our cell line model. Our results show that the phosphorylation of STAT1 is dependent on PKR activity. This finding is supported by several other studies (Hsu et al., 2004; Lee et al., 2005; Ramana et al., 2000). For example, PKR-/- bone-marrow-derived mouse macrophages are shown to exhibit defective STAT1 phosphorylation in response to LPS (Hsu et al., 2004). Furthermore, the loss of PKR activity is observed to induce the aberrant accumulation of STAT1 protein in mouse osteoblast (Yoshida et al., 2009).

It has been shown that PKR interacts with STAT1 by direct binding, although it does not appear to be a kinase-substrate interaction. Instead, the phosphorylation of STAT1 caused the dissociation of PKR –STAT1 complex (Wong et al., 1997). In addition to physical interaction, PKR has been shown to regulate STAT1 through a TC-PTP-dependent mechanism (Wang et al., 2006). PKR is able to activate TC-PTP, which in turn specifically decreases the phosphorylation of STAT1 tyrosine 701 (Wang et al., 2006). TC-PTP is also a substrate of eIF2α. Since PKR is one of the eIF2 kinases, it has been hypothesized that the induction of STAT1 dephosphorylation by TC-PTP is facilitated at least in part by the inhibition of protein synthesis as a result of PKR activation and subsequent eIF2α phosphorylation (Wang et al., 2006). Although this hypothesis is in line with our finding that tyrosine 701 is more strongly phosphorylated in cells expressing misfolded HLA-B27, in which PKR related signaling might be disturbed (Sahlberg et al., 2012), we observed the inhibition of PKR activity to prevent the STAT1 tyrosine 701 phosphorylation in HLA-B27-positive cells almost completely, even in unstimulated HLA-B27 cells. These contrasting results might be explained by the hypothesis that PKR activity is crucial for the survival of HLA-B27-expressing cells, and inhibition of PKR activity could be fatal to these cells. In fact, we have observed accelerated cell death in U937 cells after PKR inhibition (Sahlberg et al., 2012).

In addition to STAT1 tyrosine 701 residue, our results show that also the phosphorylation of serine 727 residue is dependent on PKR. This has been reported

also by others. Ramana et al, 2000, for example, showed that in PKR-null fibroblast the serine 727 residue was not phosphorylated (Ramana et al., 2000). Interestingly, in cell expressing the misfolding forms of HLA-B27, and in which the serine 727 phosphorylation is prolonged, the dependency on PKR activity was not detected until later time points. It has been shown that in stress conditions the phosphorylation of STAT1 serine 727 residue is mediated through the p38 MAPK pathway (Kovarik et al., 1999). Although it is possible that shortly after LPS stimulation or infection the phosphorylation of serine 727 is mediated through the p38 MAPK signaling pathway, we did not see the inhibition of p38 MAPK to affect the phosphorylation of serine 727 residue upon LPS stimulation or infection. Hence, instead of p38 MAPK, it could be plausible that the altered regulation of PKR we detected in HLA-B27-expressing cells (Sahlberg et al., 2012) modifies the interaction between STAT1 and PKR causing the prolonged activation of serine 727 upon *Salmonella* infection.

Our microscopy studies revealed that the inhibition of PKR did not prevent the nuclear localization of STAT1 in HLA-B27-expressing cells although it did prevent the STAT1 tyrosine 701 phosphorylation of STAT1. Together these results may indicate that the serine residue 727 could take part in the intracellular localization of STAT1.

6.6 HuR expression in HLA-B27-expressing cells

PKR is one of the regulators of HuR. It triggers the HuR cleavage under stressful conditions in its unphosphorylated state (von Roretz and Gallouzi, 2010). We detected earlier that PKR is overexpressed but hypophosphorylated in HLA-B27-positive cells (Sahlberg et al., 2012). Thus the altered PKR activation in HLA-B27-expressing cells may have an effect on HuR expression and cleavage. Indeed, we detected increased expression of full-length HuR after the inhibition of PKR in vector transfected control cells and in cell expressing the non-misfolding form of HLA-B27, but not in cells expressing misfolding HLA-B27 molecules. This implies that PKR dependent HuR cleavage seems to be disturbed in cells expressing the misfolding forms of HLA-B27. Although cells expressing misfolding HLA-B27 molecules express more PKR than control cells (Sahlberg et al., 2012), these cells expressed less HuR-CP1 and HuR-CP2 than mock and non-misfolding HLA-B27 mutated cells. Since HuR cleavage products are promoting cells´ apoptotic functions, these results indicate that the cell survival might be enhanced in HLA-B27-expressing cells. In fact, we have detected that LPS stimulated U937 cells expressing HLA-B27 are more tolerant to cell death (Sahlberg et al., 2012).

Another molecule involved in HuR regulation is p38 MAPK. Activation of p38 MAPK has shown to cause accumulation of HuR in the cytoplasm (Lafarga et al., 2009; Lin et

al., 2011). According to our earlier results, the function of p38 MAPK is dysregulated in HLA-B27-positive U937 cells (Sahlberg et al., 2007). Like with the PKR inhibitor, we saw the inhibition of p38 MAPK to influence the expression of full-length HuR in mock cells and in non-misfolding HLA-B27-expressing cells, but not in the cells expressing misfolding HLA-B27 molecules. Thus, it can be concluded that p38 MAPK is important for the regulation of HuR, but in HLA-B27-positive cells the dysregulation of p38 MAPK is likely to cause disturbances in HuR regulation as well.

Our research group's earlier results show that the survival of *S. enteritidis* is prolonged in HLA-B27-positive U937 cells (Laitio et al., 1997; Penttinen et al., 2004b). PKR is shown to regulate *salmonella*-induced cell death in p38 dependent manner (Hsu et al., 2004) and the function of both of these signaling molecules seems to be altered in U937 monocytic cells expressing HLA-B27 (Sahlberg et al., 2007; Sahlberg et al., 2012). Both PKR and p38 MAPK are involved in HuR regulation as well (Lafarga et al., 2009; Lin et al., 2011; von Roretz and Gallouzi, 2010). In addition, HuR stabilizes the mRNA of C/EBPβ (Bergalet et al., 2011), which is shown to regulate the survival of salmonella in mouse macrophages (Tanaka et al., 1995). Interestingly, we have earlier detected the overexpression of C/EBPβ in U937 HLA-B27-expressing cells (Sahlberg et al., 2012). HuR stabilizes mRNA and regulates the translation of many factors involved in inflammation and cell stress, and it directly promotes apoptosis (Katsanou et al., 2005; Mazroui et al., 2008; von Roretz et al., 2011). Hence, these modifications in the functions of p38 MAPK, PKR, C/EBPβ and HuR could create a more favorable intracellular environment for bacteria to survive and replicate in HLA-B27-expressing cells.

6.7 Cytokine signaling in HLA-B27-expressing cells

6.7.1 IL-6 signaling

Our results show that the monocytic cells expressing HLA-B27 molecule, which has a tendency to misfold, secrete substantially more IL-6 than the controls cells. According to literature, HLA-B27-positive patients suffering from SpA tend to have elevated IL-6 serum levels (reviewed in (Londono et al., 2012). Furthermore, patients with factors associated with poor prognosis, such as HLA-B27, inflammatory lower back pain or arthritis, have significantly elevated IL-6 serum levels compared with patients without these factors (reviewed in Londono et al., 2012). However, there is also a report showing that LPS stimulated peripheral blood mononuclear cells derived from HLA-B27-positive individuals with the history of ReA secret less IL-6 than HLA-B27-positive individuals with no history of ReA and less than HLA-B27-negative patients with no history of ReA (Välimäki et al., 2013).

Several different factors are involved in IL-6 production, some of which are also associated with the ER and the ER stress response, UPR. For example, the transcription factor X-box binding protein 1 (XBP-1) is known to regulate UPR (reviewed in Yoshida, 2007), but is also involved in controlling the IL-6 production (Iwakoshi et al., 2003). Moreover, XBP-1-deficient macrophages are shown to have impaired IL-6 production upon TLR agonists or infection with certain intracellular pathogen (Martinon et al., 2010). Active XBP-1 is created by unconventional splicing of its mRNA in response to misfolded or unfolded proteins in the ER (reviewed in Yoshida, 2007). Thus, the enhanced IL-6 production in HLA-B27-expressing cells could occur via misfolding induced activation of XBP-1. However, Goodall et al, 2007 has shown that HLA-B27 does not modulate the XBP-1 mRNA splicing after LPS stimulation in U937 cells (Goodall et al., 2007). Moreover, the activation of XBP-1 downstream of TLR2 and TLR4 does not seem to contribute to the ER stress response (Martinon et al., 2010).

Another molecule associated with both, the IL-6 production and ER stress, is NF-κB. NF-κB is actually one of the main regulators of IL-6 production (reviewed in Rincon, 2012). Interestingly, our research group´s earlier results show that in LPS stimulated HLA-B27-expressing cells the activation of NF-κB is faster and prolonged compared with control cells (Penttinen et al., 2002). Since the adaptation to a chronic ER-stress (which could be the case in cell expressing HLA-B27) is known to be associated with the NF-κB pathway (Bridges et al., 2006) the increased IL-6 activation in HLA-B27-expressing cells could be influenced by the enhanced NF-κB activation observed earlier these cells.

One additional molecule known to be involved in IL-6 production is C/EBPβ (reviewed in Akira and Kishimoto, 1992). It has been shown that PKR regulates C/EBPβ (Calkhoven et al., 2000). Interestingly, in addition to altered PKR regulation, the expression of C/EBPβ is observed to be increased in HLA-B27-expressing cells, even though the expression of C/EBPβ was seen to be only modestly dependent on PKR in HLA-B27-expressing cells (Sahlberg et al., 2012). Now, our results indicate that the secretion of IL-6 is completely dependent on PKR. Hence, it is possible that the overexpression of C/EBPβ in HLA-B27-expressing cells induce the increased secretion of IL-6. Although, this theory does not explain why IL-6 secretion was observed to be dependent on PKR in all cell lines we studied. The importance of PKR in IL-6 production is also reported by others. Goh and Williams, 2000, reported that the LPS induced IL-6 production was impaired in PKR-null mice (Goh et al., 2000). In 2008, Cabanski et al, showed the inhibition of PKR phosphorylation to severely impair the IL-6 production in alveolar macrophages upon LPS challenge (Cabanski et al., 2008). Furthermore, it has been shown that PKR inhibition decreases the *mycobacteria*-induced IL-6 production (Cheung et al., 2005), and overexpression of dominant negative PKR mutant prevents the

IL-6 production induced by *Herpes simplex* virus (Paludan, 2001). Interestingly, Yoshida et al, 2009 observed that in osteoblastic cells derived from dominant negative PKR mouse the higher levels of STAT1 stimulated IL-6 mRNA expression significantly, suggesting that PKR might interact with STAT1 and IL-6 pathways to regulate the differentiation of osteoblasts under immune disease or inflammation (Yoshida et al., 2009). Furthermore, it has been reported that HuR stabilizes IL-6 mRNA (Shi et al., 2012; Zhou et al., 2007). So, the increased expression of HuR in cells expressing misfolding HLA-B27 molecules, could explain the elevated IL-6 production in these cells. However, it has been reported that the mRNA stabilization of IL-6 is induced by TNF, which production, according to our experiments, is not elevated in HLA-B7-expressing cells.

Because PKR is also shown to be important in regulating the NF-κB pathway (Cabanski et al., 2008), one possible explanation for nonexistent IL-6 secretion after PKR inhibition is that inhibition of PKR may interfere with NF-κB activity and therefore IL-6 secretion as well. On the other hand, Paludan et al, 2001 showed evidence that the mechanism how NF-κB contributes to the induction of IL-6 could be independent of PKR (Paludan, 2001).

6.7.2 TNF and IL-10 signaling

The balance between the proinflammatory and the anti-inflammatory cytokine production is important in regulating effective immune response in inflammation. TNF is known to be an important mediator in the development of many autoimmune diseases. TNF-deficient animals, for example, are not able to defense efficiently against certain intracellular bacteria (reviewed in Wajant et al., 2003). There is a correlation between HLA-B27 and TNF, and both high and low TNF production is shown to be characteristic of HLA-B27 positivity and patients with SpA (Braun et al., 1999; Repo et al., 1988). TNF secretion is inhibited by a major anti-inflammatory cytokine IL-10 (reviewed in Donnelly et al., 1999). Also IL-10 is associated with the development of autoimmune diseases and SpAs. IL-10 knockout mice spontaneously develop inflammatory bowel disease and are more susceptible to CIA (Asseman et al., 1999; Johansson AC et al., 2001). Elevated IL-10 levels are found also in patients with ReA and other SpAs (Claudepierre et al., 1997; Yin et al., 1997).

In our cell line model, we did not detect differences in TNF or IL-10 production between HLA-B27-expressing cells and control cells. On the other hand, our results show a strong dependency of TNF and IL-10 production to PKR. The dependency of these cytokines to PKR is also reported by others. For example, Cabanski et al, 2008 showed that the activation of PKR by phosphorylation is critically involved in TNF secretion (Cabanski et al., 2008). Furthermore, PKR is shown to be important mediator

of latent membrane protein 1 (LMP-1) induced IL-10 and IL-6 expression (Lin et al., 2010).

The p38 MAPK signaling pathway is known to participate in TNF and IL-10 production (Campbell et al., 2004; Dobreva et al., 2009). Interestingly, we detected a strong dependency of IL-10 secretion, but not TNF secretion, to p38 MAPK. Although we have detected earlier that the signaling pathways of PKR and p38 MAPK are altered in HLA-B27-expressing cells (Sahlberg et al., 2007; Sahlberg et al., 2012), the expression of HLA-B27 molecule is not likely to influence the cytokine secretion in these cells since the secretion of these cytokines is similar in both HLA-B27-expressing cells and in control cells.

HuR is a known regulator of TNF by stabilizing its mRNA (Dean et al., 2001), whereas IL-10 is shown to suppress the p38 MAPK activation and the expression of HuR in U937 monocytic cells (Krishnamurthy et al., 2009; Rajasingh et al., 2006). Hence, there is a possibility that alterations we saw in HuR expression in cells expressing misfolding HLA-B27 molecule could have an influence on TNF and IL-10 secretion or vice versa. However, this is unlikely since we did not detect differences in the secretion of these cytokines between HLA-B27-expressing cells and control cells. In addition, although the inhibition studies with PKR and p38 inhibitors proved that both TNF and IL-10 productions are strongly dependent on PKR and that IL-10 production is dependent on p38 MAPK as well, the dependency of TNF and IL-10 on PKR and p38 MAPK were equal in all cell lines studied.

6.8 Methodological considerations

This study was carried out with transfected human U937 monocytic cell line cells to study the effects of HLA-B27 expression on signaling molecules. U937 cells are premature monocytic cells, which lacks the expression of typical monocyte/macrophage-specific markers such as CD14 and MHC class II molecules (Hass et al., 1989). To make the cells resemble more mature macrophage-like cells, they were pre-stimulated with PMA.

Using a cell line model has both advantages and disadvantages, which should be taken in to consideration when analyzing the results. For example, transfected cell line model ideally offers a model where cells differ from each other only by the expression of transfected genes and the proteins they encode. Hence, the interindividual variations due to the differences in the genetic background, which may disturb the experiments performed with freshly isolated primary cells, can be avoided. Whereas the availability

of high number of cells and the possibility to control the experimental set up are practical strengths of the cell lines.

However, cell line cells are immortalized cancer cells, in which the functions like intracellular signaling mechanisms involved in inflammatory responses, may differ from those of normal cells. Nevertheless, when the effects of transfected genes on cells which are otherwise similar are studied, the comparison is still possible. There is also a possibility that the transfection itself may alter the functions of the cells by inducing or suppressing the expression of genes when foreign DNA is inserted in to the genome. In addition, the heterogeneity of cell population and the transfection may result in the selection of the clones that differ from each other by some other way than the transfected genes. Furthermore, it should be also noted that there is no interaction between different cell types like in more complex in vivo situations.

To overcome some of the disadvantages the cell line model presents, we used several independent cell lines carrying HLA-B27 transgene (genomic clone of HLA-B27, cDNA of HLA-B27 and cells with HLA-B27 mutations) and several control cell lines as well. The cells were selected for the positive cell surface expression of the transfected genes, which were expressed at the same physiological level as the endogenously expressed MHC class I molecule HLA-B51 (Laitio et al., 1997). Furthermore, we took the new batch of cells from the freezer every 3 months to avoid mutations and other changes, which the maintenance of the cell line for a long period of time can predispose the cells to.

7. SUMMARY AND CONCLUSIONS

A strong association of HLA-B27 with autoimmune disease group of SpAs is known for decades. Despite the intensive research, the exact mechanism how HLA-B27 causes susceptibility to these diseases is still not known. ReA is one of the SpA diseases. It is usually triggered by infection caused by certain gram-negative bacteria like *Salmonella, Yersinia* or *Chlamydia*. It has been shown that in HLA-B27-expressing cells the triggering bacteria are able to survive longer than in control cells. In addition, these bacteria are able to replicate inside of HLA-B27-expressing cells. HLA-B27 molecule has some unique features. For example, HLA-B27 HCs have a tendency to misfold during assembly in the ER. Misfolded HLA-B27 molecules may accumulate in the ER, which can cause ER stress. It has been suggested that the misfolding feature of HLA-B27 is one of the factors contributing to its pathogenicity.

The purpose of this study was to reveal some of the mechanisms how the expression of HLA-B27 molecule could contribute to the development of disease susceptibility in SpAs. In more detail, we aimed to study whether HLA-B27 molecule alters the activation of such signaling molecules and/or signaling cascades, which are involved in inflammatory reactions and possibly enabling bacteria to survive and replicate inside HLA-B27-expressing monocytes. There are evidences that some signaling cascades are disturbed in HLA-B27-expressing cells. For example, the activation of PKR is altered in HLA-B27-expressing human U937 monocytic cells.

In this study, we studied the signaling molecule STAT1, which is a transcription factor mediating the anti-bacterial and anti-viral signals of IFNs. We discovered that the activation of STAT1 is enhanced in HLA-B27-expressing cells, which has a tendency to misfold. The phosphorylation of STAT1 tyrosine 701 residue, which is needed and sufficient for its dimerization, nuclear translocation and DNA binding, was enhanced in HLA-B27-expressing U937 cells, even without any external trigger (like LPS). In addition, the phosphorylation of the other important phosphorylation site of STAT1, serine 727, was prolonged in HLA-B27-expressing cells. Phosphorylation of STAT1 serine 727 is needed for the maximal induction of STAT1´s transcriptional activity and is shown to be important in bacterial clearance. These finding are functionally relevant since according to localization studies with confocal microscopy more STAT1 was localized in the nucleus of HLA-B27-expressing cells compared with the control cells. Furthermore, the activation of STAT1 was dependent on PKR.

The enhanced and prolonged activation of STAT1 could lead to an overexpression of IFN-γ-dependent genes, as well other STAT1-dependent genes, causing exaggerated

inflammatory responses in HLA-B27-expressing cells. In fact, it has been reported that HLA-B27-positive patients have more severe and prolonged symptoms than HLA-B27-negative patients. Moreover, we found out that the secretion of one of the most important cytokines involved in the inflammatory response, IL-6, is elevated in HLA-B27-expressing cells. In addition, we discovered that the expression of posttranscriptional gene regulator HuR was altered in HLA-B27-expressing cells. Furthermore, our results indicate that the tendency of HLA-B27 HC to misfold is an important factor in altered inflammatory responses detected in HLA-B27-positive cells. This finding confirms further one of the dominant theories of HLA-B27 HC misfolding playing an important part in the pathogenesis of HLA-B27.

Altogether, these results may offer an explanation why HLA-B27-positive patients suffering from SpA tend to develop more severe and exaggerated inflammatory reactions and symptoms than HLA-B27-negative patients. Better understanding of the mechanisms how HLA-B27 causes susceptibility to these diseases will help to develop more specific and effective treatment against ReA and other SpAs in future.

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