THE ROLE OF GALECTIN-3 AND GALECTIN-9 IN THE CHRONIC INFLAMMATION OF RHEUMATOID ARTHRITIS

by

Magdalena Anna Bik

A thesis submitted to
The University of Birmingham
for the degree of
DOCTOR OF PHILOSOPHY

School of Immunity and Infection
College of Medical and Dental Sciences
The University of Birmingham
August 2009

UNIVERSITY OF
BIRMINGHAM

University of Birmingham Research Archive

e-theses repository

This unpublished thesis/dissertation is copyright of the author and/or third parties. The intellectual property rights of the author or third parties in respect of this work are as defined by The Copyright Designs and Patents Act 1988 or as modified by any successor legislation.

Any use made of information contained in this thesis/dissertation must be in accordance with that legislation and must be properly acknowledged. Further distribution or reproduction in any format is prohibited without the permission of the copyright holder.

Abstract

Fibroblasts are important regulators of inflammatory processes. The phenotypes of fibroblasts differ according to anatomical site which may determine immune functions such as leukocyte accumulation and predilection for inflammatory disease in certain tissues.

This thesis describes the expression profile and explores the function of a family of immunomodulatory proteins (galectins) in fibroblasts from rheumatoid arthritis patients. Synovial fibroblasts were found to differ significantly from bone marrow and skin fibroblasts with higher expression of galectin-9 and galectin-12 in synovial fibroblasts.

Galectin-9 and galectin-3 expression was also examined in situ in synovial tissue from rheumatoid arthritis (RA) and osteoarthritis (OA) patients. Expression of both galectins was higher in RA synovial tissue compared to OA but not in synovial fibroblasts cultured in vitro. Galectin-3 expression seemed to be controlled by epigenetic factors (methylation) but not cytokine stimulation. Galectin-9 production was up-regulated by interferon-γ, interleukin-1β and ligands for Toll-like receptors 3 (TLR3) and 4 (TLR4). It was found that intracellular presence of galectin-9 in RA synovial fibroblasts increased their resistance to apoptosis.

Galectin-3 levels are increased in the joints of patients with rheumatoid arthritis. Studies on the effect and mechanism of galectin-3 action on fibroblasts revealed that exogenously added galectin-3 induced production of cytokines (IL-6) from synovial and skin fibroblasts but the production of monocyte attracting chemokines (CCL5, CCL2) was induced uniquely in fibroblasts derived from the synovium. Different signalling pathways mediated the secretion of those mediators. IL-6 release depended on MAP kinases p38, ERK and JNK as well as NFκB transcription factor, whereas CCL5 production required PI3K/Akt and NFκB.

Acknowledgements:

I would like to thank my supervisors, Professor Janet Lord for her guidance and encouragement throughout my studies and Professor Christopher Buckley whose enthusiastic approach to research has always been an inspiration.
Dr John Curnow and Dr Karim Raza gave me valuable, if slightly critical, comments and advice during my progress reports. Many thanks to Dr Andrew Filer for a very productive collaboration (i.e. overloading me with work at times).
I would also like to acknowledge all the members of Immunology group who helped me along the way: Hema Chahal for technical support as well as taking care of my staying fit, Dr Jason Lee who has been the most helpful colleague ever, Dr See Heng Wong who introduced me to the lab work during my first months, Dr Keqing Wang for on-site phlebotomy services and Dr Peter Hampson.
I am equally thankfull to the Rheumatology group, Kath Howlett for providing us with fibroblasts, Steve Kissane for answering all PCR-related questions, Debbie Hardie for help with confocal microscopy. Finally, all colleagues from the 3rd floor write-up (or rather chit-chat) area for creating the friendly and fun environment.

My special thanks go to Professor Steffen Gay who hosted me at the Centre for Experimental Rheumatolgy (Univeristy Hospital, Zurich) for several months and to his group. I am especially gratefull to Caroline Ospelt, my 'Götti' ('god father') for guiding me through the new lab, Joanna Stanczyk and Astrid Jungel for valuable discussion, Maria Comazzi – the queen of immunohistochemistry and Emmanuel Karouzakis whose greek philosophy always helped me to overcome the frustrations of daily experimental failures. I had the most amazing, though very busy time in that lab (and outside of it in the nearby Alps).

I would like to acknowledge Marie Curie Actions for funding this work.

Finally, I would like to thank my family, especially my mum, for not letting me give up at difficult times and always believing in me.

Abbreviations:

AEC – 3-amino-9-ethylcarbazole
AGE – advanced glycation end-products
AP – alkaline phosphatase
AP-1 – activator protein-1
APC – antigen presenting cell
BCA – B cel attracting chemokine
Bcl – B cell lymphoma
bLP – bacterial lipopeptide
BM – bone marrow
BSA – bovine serum albumine
CBP – carbohydrate binding protein
CCL – chemokine (C-C motif) ligand
CCP – cyclic citrullinated peptide
CD – cluster of differentiation
cDNA – complimentary deoxyribonucleic acid
CIA – collagen-induced arthirits
CLC – Charcot-Leyden crystal protein
CpG – deoxy-cytidylate-phosphate-deoxy-guanylate
CRD – carbohydrate recognition domain
CRP – C-reactive protein
CTLA4 – cytotoxic T lymphocyte antigen
CXCL – chemokine (C-X-C motif) ligand
DF – dermal fibroblasts (skin)
DAB – diaminobenzidine
DMARDs – disease-modifying antirheumatic drugs
DMSO – dimethylsulphoxide
DNMT – DNA methyl transferase
EAE – experimental autoimmune encephalitis
ECL – enhanced chemiluminescence
ECM – extracellular matrix
EDTA – ethylenediamine-tetraacetic acid
EGF – epidermal growth factor
ELISA – enzyme-linked immunosorbent assay
ER – endoplasmic reticulum
ERK – extracellular signal-regulated kinase
FACS – fluorescence activated cell sorting
FADD – Fas-associated death domain
FCM – fibroblast-conditioned medium
FcR – receptor for Fc portion of immunoglobulin
FCS – fetal calf serum
FDC – follicular dendritic cell
FGF – fibroblast growth factor
FITC – fluorescein isothiocyanate
FLICE – FADD-like interleukin-1 beta-converting enzyme
FLIP – FLICE-inhibitory protein
FLS – fibroblast-like synoviocyte

FS – forward scatter
GC – germinal centre
G-CSF – granulocyte colony stimulating factor
GI – gastro-intestinal tract
GM-CSF – granulocyte-macrophage colony stimulating factor
GRO – growth regulated oncogene
HLA-DR – human leukocyte antigen-DR
HRP – horseradish peroxidase
HSP – heat-shock protein
HTLV-1 – human lympotropic virus-1
HUVEC – human umbilical vein endothelial cells
ICAM – intracellular cell adhesion molecule
IGF – insulin-like growth factor
IgG – immunoglobulin G
IFN – interferon
IκB – inhibitor of nuclear factor-kappa B
IL – interleukin
JAK – Janus kinase
JNK – c-Jun N-terminal kinase
LBP – non-integrin laminin binding protein
LPS – lipopolysaccharide
LT – lymphotoxin
MAPK – mitogen-activated protein kinase
MCP-1 – monocyte chemoattractant protein-1
MEK – MAP/ERK kinase
MHC – major histocompatibility comples
MIP-1 – macrophage inflammatory protein-1
MMP – matrix metalloproteinase
mRNA – messenger ribonucleic acid
MT-MMP – membrane type matrix metalloproteinase
NADPH – nicotinamide-adenosine dinucleotide phosphate
NF-κB – nuclear factor kappa B
NSAIDs – nonsteroidal anti-inflammatory drugs
OA – osteoarthritis
OD – optical density
ODF – osteoclast differentiation factor
PA – plasminogen activator
PAGE – polyacrylamide gel electrophoresis
PADI4 – peptidylarginine deiminase 4
PBS – phosphate buffered saline
PCR – polymerase chain reaction
PDGF – platelet-derived growth factor
PE – phycoerythrin
PGE – prostaglandin E
PI – propidium iodide
PKC – protein kinase C
PS – phosphatidylserine
PTEN – phosphatase and tensin homolog

PTPN22 – protein tyrosine phosphatase N type 22
PVDF – polyvinylidene difluoride
RA – rheumatoid arthritis
RAGE – receptor for advanced glycation end-products
RANK – receptor activator of NF κB
RANTES – Regulated upon Activation, Normal T-cell Expressed, and Secreted
RF – rheumatoid factor
RNA – ribonucleic acid
RNP – ribonucleoprotein
rpm – revolutions per minute
SAPK – stress-activated protein kinase
SCID – severe combined immunodeficiency
SD – standard deviation
SDF-1 – stromal derived factor-1
SDS – sodium dodecyl sulphate
SEM – standard error of the mean
SF – synovial fluid
siRNA – small interfering RNA
SS – side scatter
STAT – signal transducer and activator of transcription
STP – staurosporine
SY – synovial fibroblasts
Syk – spleen tyrosine kinase
TBS – Tris buffered saline
TCR – T cell receptor
TGFβ – transforming growth factor beta
Th – T helper lymphocyte
TIM – T cell immunoglobulin mucin
TLR – Toll-like receptor
TMB – tetramethylbenzidine substrate
TNFα – tumor necrosis factor alpha
UV – ultraviolet
VCAM – vascular cell adhesion molecule
VEGF – vascular endothelial growth factor
VLA – very late antigen
vWF – von Wildebrand factor

LIST OF FIGURES AND TABLES

Fig.1.1 Structure and classification of human galectins…………………………..................................1

Table 1.1 Tissue distribution of galectins………………………………………….................................5

Fig.1.2 Examples of the biological actions of galectins…………………………..................................7

Fig.1.3 Schematic representation of joint structure in health and disease……….................................21

Table 2.1 Antibodies used in the study……………………………………...….................................43

Table 2.2 Sequences of primers for PCR…………………………………….....................................50

Table 2.3 Recombinant proteins used in neutrophil survival assays…………….................................58

Fig.2.1 Gating of neutrophils based on their forward scatter vs. side scatter profile………………….59

Fig.2.2 Identification of apoptotic cells using DiOC6 staining…………………..................................59

Fig.3.1 Effect of conditioned media from cytokine-activated fibroblasts on neutrophil survival in culture……………………….................……………………………………………………………64

Fig.3.2 Effect of GM-CSF depletion on neutrophil survival…………………..................................65

Fig.3.3 Blocking of IFN receptors on neutrophils -effect on survival induced by recombinant IFNβ and fibroblast-conditioned media……………….………...68

Fig.3.4 Interleukin-6 levels in fibroblast-conditioned media……………....………...........................69

Fig.3.5 Effect of recombinant IL-6 on neutrophil survival………………...……..........................69

Fig.3.6 Blocking of IL-6 receptors on neutrophils -effect on survival induced by recombinant IL-6 and fibroblast-conditioned media…………………………...70

Fig.3.7 Effect of recombinant TNFα and depletion of TNFα and/or GM-CSF from fibroblast-conditioned media on neutrophil survival…………………………...72

Fig.3.8 Effect of recombinant galectin-3 on neutrophil survival and levels of galectin-3 in fibroblast-conditioned media……………………………….…...74

Fig.4.1 Expression profile of galectins in fibroblasts of different origin……….................................85

Fig.4.2 Quantitative differences in galectins mRNA expression in RA fibroblasts. ………………...86

Fig.4.3 Galectin-3 and -9 expression in fibroblasts from RA and OA patients…..............................88

Fig.4.4 Immunohistochemical detection of galectin-3 in synovial tissues from RA and OA patients-fibroblasts………………………………………………...90

Fig.4.5 Detection of galectin-3 in RA synovial tissue – macrophages………….................................91

Fig.4.6 Immunohistochemical detection of galectin-9 in synovial tissue from RA and OA patients-fibroblasts………………………………………………...92

Fig.4.7 Detection of galectin-9 in RA synovial tissue – macrophages………….................................93

Fig.4.8 Immunohistochemical detection of galectin-9 in synovial tissue from RA and OA patients-endothelial cells……………………………………………..94

Fig.4.9 Galectin-9 in RA fibroblasts at sites of invasion……………………….................................95

Fig.4.10 Effect of cytokine stimulation on expression of galectin-3 and -9 mRNA. ……………….98

Fig.4.11 Galectin-9 protein in synovial fibroblasts after IFNγ treatment………...............................101

Fig.4.12 Activation of STAT and p38 MAPK in synovial fibroblasts stimulated with IFNγ……….101

Fig.4.13 Translocation of STAT in synovial fibroblasts stimulated with IFNγ..................................102
Fig.4.14 Effect of TLR-ligands on expression of galectin-3 and -9 mRNA……................................105
Fig.4.15 Schematic representation of galectin-3 and galectin-9 promoters……................................108
Fig.4.16 Effect of DNA demethylation on expression of galectin-3 and -9 protein. ………………..109
Fig.5.1 Galectin-9 silencing in RA synovial fibroblasts……………………….................................122
Fig.5.2 Effect of galectin-9 silencing on synovial fibroblast apoptosis……….................................123
Fig.5.3 Effect of galectin-9 silencing on synovial fibroblasts proliferation……...............................125
Fig.5.4 Effect of galectin-9 silencing on production of IL-6, MMP-3, IL-8 and CCL5 by synovial fibroblasts………………………………...…126
Fig.6.1 Galectin-induced production of cytokines from fibroblasts……………..............................132
Fig.6.2 Galectin-3 - induced production of IL-6 and CCL5 is concentration dependent……………133
Fig.6.3 Effect of neutralizing TNFα on galectin-3 induced IL-6, CCL2 and CCL5 production…….135
Fig.6.4 Diagram of potential galectin-3 signalling pathways and inhibitors used to examine them...136
Fig.6.5 Inhibition of IL-6 production in galectin-3 stimulated synovial and skin fibroblasts……….138
Fig.6.6 Inhibition of CCL5 production in galectin-3 stimulated synovial fibroblasts……………….139
Fig.6.7 Activation of protein kinases following galectin-3 treatment in synovial and skin fibroblasts………………………………………………………..141
Fig.6.8 Activation of NFκB transcription factor in synovial and skin fibroblasts. ………………….142
Table 7.1 Current primary medications used to treat RA……………………...............................152

TABLE OF CONTENTS

1.1 GALECTINS 1
1.1.1 Structure and specificity *1*
1.1.2 Cellular localization and tissue distribution *3*
1.1.3 Functions of galectins *5*
1.1.4 Role of galectins in immunomodulation *10*
1.1.4.1 Galectins and adaptive immunity 10
1.1.4.2 Galectins and innate immunity 12
1.1.4.3 Galectins and immune pathology 13
1.1.5 Galectin-3 .... *14*
1.1.6 Galectin-9 .... *17*
1.2 RHEUMATOID ARTHRITIS 18
1.2.1 General information *18*
1.2.2 Histopathology of RA *20*
1.2.3 Components of the inflammatory infiltrate *23*
1.3 OSTEOARTHRITIS 29
1.4 FIBROBLASTS 30
1.4.1 Fibroblasts and the immune system *30*
1.4.2 Fibroblast diversity *32*
1.5 SYNOVIAL FIBROBLASTS IN RHEUMATOID ARTHRITIS 33
1.5.1 Activation of synovial fibroblasts *33*
1.5.2 Transformed phenotype of RA synovial fibroblasts *36*
1.5.3 Synovial fibroblasts in rheumatoid arthritis and osteoarthritis *38*
1.5.4 Interactions of fibroblasts with immune cells in the rheumatoid joint *39*
1.6 AIMS OF THE THESIS 42

2.0 MATERIALS AND METHODS 43

2.1 ANTIBODIES 43
2.2 CELL CULTURES 44
2.2.1 Fibroblast culture *44*
2.2.2 Neutrophil isolation *45*
2.3 CELL TREATMENTS 46
2.3.1 Cytokines *46*
2.3.2 TLR ligands *46*
2.3.3 DNA methylation inhibitor *46*
2.3.4 Galectin-3 and inhibitors *47*
2.3.5 Fibroblast-conditioned medium production *47*
2.4 CYTOKINE DEPLETION AND BLOCKADE STUDIES 48
2.4.1 Receptor blocking on neutrophils *48*

2.4.2 Cytokine depletion from FCM ... *48*
2.5 GALECTIN RNA EXPRESSION IN FIBROBLASTS ... 49
2.5.1 Fibroblasts mRNA isolation ... *49*
2.5.2 cDNA synthesis ... *49*
2.5.3 End-point PCR ... *50*
2.5.4 Real-time PCR ... *51*
2.6 PROTEIN EXPRESSION AND SECRETION ... 52
2.6.1 Immunohistochemistry ... *52*
2.6.2 Western blotting ... *53*
2.6.3 Enzyme-linked immuno-sorbent assays (ELISAs) ... *55*
2.7 TRANSCRIPTION FACTOR ACTIVATION ... 55
2.7.1 NFκB activation - TransAM assay ... *55*
2.7.2 STAT translocation – confocal microscopy ... *56*
2.8 GALECTIN-9 SILENCING ... 57
2.9 FUNCTIONAL ASSAYS ... 58
2.9.1 Neutrophil survival assays and assessment of apoptosis ... *58*
2.9.2 Fibroblasts apoptosis assay ... *60*
2.9.3 Fibroblasts proliferation assay ... *61*
2.10 STATISTICAL ANALYSIS ... 61
RESULTS AND DISCUSSION ... 62

3.0 FIBROBLAST-INDUCED NEUTROPHIL SURVIVAL ... 62

3.1 BACKGROUND ... 62
3.2 IDENTIFICATION OF NEUTROPHIL SURVIVAL FACTORS PRODUCED BY ACTIVATED FIBROBLASTS 63
3.3 ROLE OF FIBROBLAST-PRODUCED GALECTIN-3 IN NEUTROPHIL SURVIVAL ... 73
3.4 DISCUSSION ... 75

4.0 EXPRESSION OF GALECTINS AND THEIR REGULATION IN FIBROBLASTS ... 81

4.1 BACKGROUND ... 81
4.2 EXPRESSION OF GALECTINS IN FIBROBLASTS ... 82
4.4 REGULATION OF EXPRESSION OF GALECTIN-3 AND GALECTIN-9 ... 96
4.4.1 Cytokines ... *96*
4.4.2 Toll-like receptors ligands ... *103*
4.4.3 Epigenetic factors – DNA methylation ... *106*
4.5 DISCUSSION ... 110

5.0 ROLE OF GALECTIN-9 IN RA SYNOVIAL FIBROBLAST FUNCTION ... 120

5.1 BACKGROUND ... 120
5.2 EFFECT OF GALECTIN-9 SILENCING ON FIBROBLAST APOPTOSIS AND PRODUCTION OF INFLAMMATORY MEDIATORS ... 121

5.3 DISCUSSION....127

6.0 RESPONSE OF FIBROBLASTS TO GALECTIN-3....130

6.1 BACKGROUND.... 130

6.2 DIFFERENTIAL PRODUCTION OF CYTOKINES AND CHEMOKINES FROM FIBROBLASTS STIMULATED BY GALECTIN-3.... 131

6.3 GALECTIN-3-INDUCED IL-6, CCL5 AND CCL2 PRODUCTION IS NOT MEDIATED VIA AUTOCRINE TNFA.... 134

6.4 SIGNALING PATHWAYS INDUCED IN FIBROBLASTS BY GALECTIN-3.... 136

6.5 DISCUSSION.... 143

7.0 GENERAL DISCUSSION....147

References....154

1.0 INTRODUCTION

1.1 Galectins

1.1.1 Structure and specificity

Lectins are nonenzymatic and non-immunoglobulin proteins that bind carbohydrates. Galectins (previously called S-type lectins) constitute one of the several families of endogenous lectins and are named after their ability to bind β-galactosides (Barondes et al., 1994). Galectins and galectin-like proteins are present in all animal kingdoms and are reasonably well conserved throughout evolution (Cooper and Barondes, 1999). For example, all galectins display sequence similarity in the carbohydrate-recognition domain (CRD) which consists of about 130 amino acids with a globular tertiary structure forming a groove in which carbohydrate is bound (Leffler et al., 2004).

Currently there are 15 mammalian galectins known, 11 of which can be found in humans, namely galectins 1-4, 7-10, 12, 13 and 14 (Liu and Rabinovich, 2005). They can be subdivided into three groups based on their structural organisation (Fig.1.1).

Type	Structure		Galectin
proto-type	one CRD		1, 2, 7, 10, 13,14
chimera-type	CRD + non-lectin domain		3
tandem repeat	Two-CRDs		4, 8, 9, 12

Fig. 1.1 Structure and classification of human galectins.

Galectins containing only one carbohydrate-recognition domain (CRD) are called proto-type and include galectins: 1, 2, 5, 7, 10, 11, 13, 14 and 15. Those with two distinct CRDs in tandem connected by a linker region (tandem repeat-type) are

galectin-4, 6, 9, 12. Third is a chimera-type with one CRD plus an unusual non-lectin domain rich in proline and glycine. The only representative of the latter group is galectin-3 (Rubinstein et al., 2004). Recently discovered multiple isoforms of galectin-8 confound slightly this classification as three of them belong to tandem-repeat group while other three to the prototype one (Bidon et al., 2001). There are no human counterparts of galectin-5, -6, -11 and -15 (Fig. 1.1).

The endogenous ligands for galectins are saccharide chains of glycoconjugates (i.e. glycoproteins or glycolipids). Galectins show a particular preference for glycans containing the ubiquitous disaccharide N-acetyllactosamine (Galβ1-3GlcNAc or Galβ1-4GlcNAc) (Hirabayashi et al., 2002; Patnaik et al., 2006). Most of such ligands would be found in the extracellular compartment or within the luminal spaces of cellular organelles. Considering the wide variety of glycosylated molecules there are numerous potential ligands for galectins. However, most information on the sugar-specificity of galectins comes from in vitro biochemical studies where the carbohydrate structures are not presented in their natural context of membrane-associated proteins or lipids. This presents a serious hurdle to identifying the real binding partners. However, new tools are being developed to facilitate further research (de Melo et al., 2007).

Ligands for galectin binding that have been successfully demonstrated so far comprise glycoconjugates of the extracellular matrix (ECM) such as laminin (Barboni et al., 1999), fibronectin (Ozeki et al., 1995), elastin (Ochieng et al., 1999) as well as cell surface glycoproteins including integrins (Hadari et al., 2000; Fukumori et al., 2003; Ochieng et al., 1998b), carcino-embrionic antigen (CEA) (Ohannesian et al., 1995) or bacterial lipopolysaccharides (LPS) (Gupta et al., 1997).

Other cell-type specific receptors identified for galectins are CD43 and CD45 on T cells (Hernandez et al., 2006) or CD66 on neutrophils (Feuk-Lagerstedt et al., 1999). Certain galectins can also interact with other proteins independently of their carbohydrate-binding sites which further extends the range of their potential ligands. In fact, some galectins, for example galectin-10, possess low affinity for galactosides and may have other specificities (Leffler et al., 2004).

1.1.2 Cellular localization and tissue distribution

Although galectins have features of cytosolic proteins they can be found not only inside the cells but also in the extracellular space. The mechanism of secretion is not clear since galectins lack the signal sequence required for classical secretory pathway via endoplasmic reticulum (ER) and vesicles of the Golgi apparatus (Hughes, 1999). Proposed mechanisms include membrane blebbing into exosomes which then detach from the cell and release their contents into the extracellular space (Nickel, 2003). Another possibility of crossing lipid bilayers that was described for galectin-3 is by direct interaction with phospholipids or cholesterol (Lukyanov et al., 2005). The unique N-terminal non-lectin sequence may be responsible for this phenomenon (Menon and Hughes, 1999). More recently exit of galectin-1 from the cell via interaction with specific counter receptors was demonstrated (Seelenmeyer et al., 2005). These β-galactoside-containing glycolipids would translocate between the outer and inner leaflets of plasma membrane with an attached galectin, a process catalyzed by a membrane-resident enzyme.

Localisation of galectins inside the cells can be cytoplasmic or nuclear (Wang et al., 2004; Liu et al., 2002). Galectin-9 also exhibits unusual membrane localization and was described to function as a urate transporter (Lipkowitz et al., 2004). This

indicates that despite the lack of a signal sequence characteristic for transmembrane proteins, refolding of these molecules and incorporation into cell membranes may occur.

Tissue distribution of galectins varies for different members of the family (Table 1.1). Several of them such as galectin-1, -3 or -8 are expressed in a wide range of tissues (Chiariotti et al., 2004). Others have a more restricted localization. Galectin-7 for example is characteristic only for stratified epithelia (Magnaldo et al., 1998). On the other hand, galectin-4 is confined to epithelial cells of the gastro-intestinal (GI) tract (Huflejt and Leffler, 2004). Similar localization in the GI tract was reported for galectin-2 (Sturm et al., 2004). Galectin-10, also referred to as Charcot-Leyden crystal protein (CLC), seems to be unique for eosinophilic and basophilic leukocytes (Dyer and Rosenberg, 2001) and T regulatory cells (Kubach et al., 2007).

Importantly, some galectins may be induced in pathological conditions in different cell types, for example galectin-7 in aggressive T cell lymphoma (Demers et al., 2007) or galectin-2 which was shown in atherosclerotic plaques (Ozaki et al., 2004). It has also been suggested that expression of certain galectins may be correlated with the stage of the cell cycle. Galectin-12, first identified as adipose tissue-specific (Hotta et al., 2001), was later reported in heart, pancreas, spleen, thymus, and peripheral leukocytes (Yang et al., 2001). It was particularly expressed in cells synchronized at the G1 phase or the G1/S boundary of the cell cycle.

Table. 1.1 Tissue distribution of galectins.

	tissues	cells	References
galectin-1	lymphoid organs, muscles, kidney, brain, placenta, cornea	thymic epithelial cells, neurons, T cells, macrophages, B cells	(Camby et al., 2006; Perillo et al., 1997; Blaser et al., 1998; Zuniga et al., 2001)
galectin-2	intestine, induced in atherosclerotic plaques	epithelial cells, induced in smooth muscle cells, macrophages	(Hokama et al., 2004; Ozaki et al., 2004; Hokama et al., 2008)
galectin-3	multiple organs (lung, thymus, intestine, kidney, pancreas, breast, prostate, blood cells)	epithelial cells, fibroblasts, chondrocytes, osteoblasts, keratinocytes, dendritic cells, neutrophils, basophils, eosinophils, mast cells, monocytes, macrophages, induced in lymphocytes	(Dumic et al., 2006; Openo et al., 2000)
galectin-4	alimentary tract (particularly intestine and colon); induced in epithelial cancers	epithelial cells	(Huflejt and Leffler, 2004; Rechreche et al., 1997; Kondoh et al., 1999)
galectin-7	stratified epithelia (epidermis, tongue, esophagus)	keratinocytes, epithelial cells, induced in T cell lymphoma	(Madsen et al., 1995)
galectin-8	ubiquitous		(Gopalkrishnan et al., 2000; Bidon et al., 2001)
galectin-9	peripheral blood leukocytes	T cells, induced in fibroblasts, endothelial cells	(Tureci et al., 1997; Matsumoto et al., 1998; Imaizumi et al., 2002)
galectin-10		eosinophils, Treg cells	(Dyer and Rosenberg, 1996; Kubach et al., 2007)
galectin-12	adipose tissue, heart, pancreas, spleen, thymus,	adipocytes, leukocytes	(Hotta et al., 2001; Yang et al., 2001)
galectin-13	placenta		(Yang et al., 2002)

1.1.3 Functions of galectins

Depending on their particular structure, galectins can act as bivalent or multivalent carbohydrate-binding molecules. In addition to those already having two binding sites, one-CRD galectins form non-covalent dimers, while galectin-3 aggregates into oligomers via its tandem-repeat region (Ahmad et al., 2004). Such aggregation appears to be required for biological activity since monomeric forms do not induce

an intracellular signal despite binding to the cells (Dias-Baruffi et al., 2003; Karlsson et al., 1998).

The multivalent structure of galectins allows them to bind different carbohydrate moieties of molecules present on the cell surface and in the extracellular matrix. In this way galectins can mediate cell adhesion, migration and cell-cell interactions (Liu and Rabinovich, 2005). On the other hand cross-linking of glycoreceptors within the same cell can initiate intracellular signal transduction (Fig. 1.2). The ability to reorganize membrane glycoproteins into lipid rafts may have implications in signalling events not directly induced by galectins such as TCR signalling (Miceli et al., 2001; Demetriou et al., 2001). It also prevents endocytosis of receptors trapped in such surface lattices thereby regulating cell responsiveness to stimuli (Rabinovich et al., 2007). In this respect it is interesting to note that many receptors for growth factors (eg. epidermal-EGFR, fibroblast-FGFR, insulin-like-IGFR, platelet-derived-PDGFR) are rich in N-glycans that are targets for binding by galectins (Lau et al., 2007).

In addition to their extracellular roles, galectins can also act inside the cell (Fig.1.2) participating in RNA processing in the nucleus (Wang et al., 2004) or regulating cell cycle progression (Yang et al., 2001b). Another example is modulation of apoptosis via interaction with Bcl-2 family members in the cytoplasm (Yang et al., 1996; Liu et al., 2002). These functions of galectins are typically independent of their sugar-binding activities and rely rather on protein-protein interactions (Rabinovich et al., 2002). Interestingly, the same galectin (e.g. galectin-3) may exert different and opposing effects on a cell depending on whether it acts from the outside or inside of the cell (Nangia-Makker et al., 2007; Liu and Rabinovich, 2005). In the first case it

can trigger cell death (Rubinstein et al., 2004), in the latter it protects the cell from apoptosis (Hoyer et al., 2004).

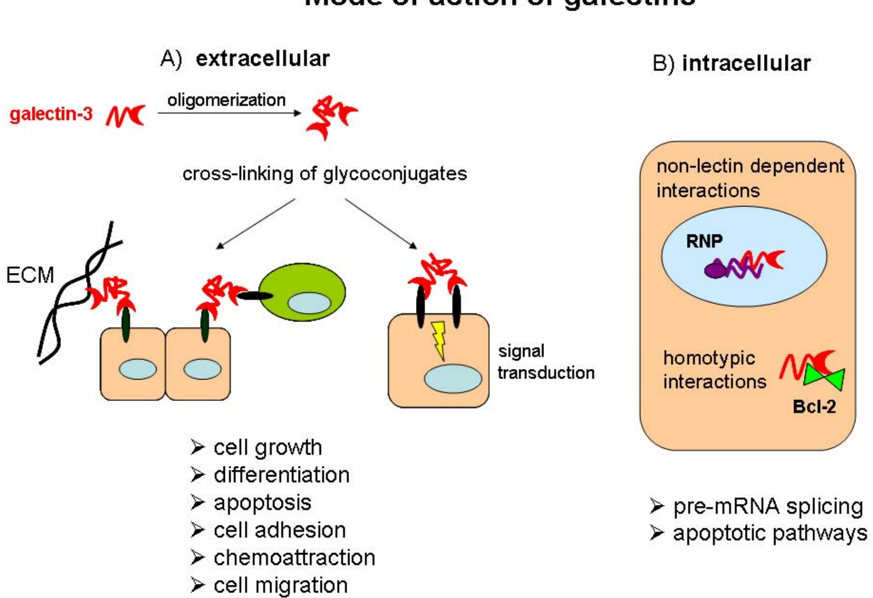


Fig. 1.2 Examples of the biological actions of galectins

The figure shows mechanisms of action of galectins (cross-linking surface molecules or direct protein-protein interactions) as exemplified by galectin-3. Biological effects regulated by galectins range from cell adhesion and migration to cell growth, differentiation, activation and finally apoptosis.

Adhesion and migration of cells requires coordinated recognition and binding to extracellular matrix (ECM) components. Many of these glycoproteins including fibronectin and laminin are recognized by galectins. The presence of galectins may facilitate cell adhesion by bridging and cross-linking adhesion molecules with the matrix or, conversely, cause detachment by saturating the available binding sites

(Elola et al., 2007). Binding of galectin-1 or galectin-8 to β1-integrin was shown to modulate cell adhesion to laminin and trigger cytoskeleton reorganization (Moiseeva et al., 2003). Moreover, engagement of integrins on the cell surface can trigger signals regulating cell fate. For example, inhibition of cell adhesion caused by galectin-8 induces apoptosis of human carcinoma (Hadari et al., 2000). On the other hand, enhanced attachement to extracellular-matrix proteins mediated by galectin-3 may protect certain cells from death (Matarrese et al., 2000). Galectins can also regulate cell-cell interactions as exemplified by an enhancement of dendritic cells and naïve T cells interactions in lymph nodes (Swarte et al., 1998).

Many observations implicate a role for galectins in growth regulation of various cells. Expression of galectin-3, for example, is related to the proliferative state of the cells with marked upregulation in proliferating cells (Yang and Liu, 2003). Their growth-promoting effect occurs through induction of cyclin D1 and c-myc oncogene (Yoshii et al., 2002; Shimura et al., 2004). Galectins also exert cytostatic effects. Galectin-1 can inhibit antigen-induced proliferation of T cells (Blaser et al., 1998). In another study, however, it displayed a biphasic effect on cell proliferation which was dependant upon its concentration (Adams et al., 1996). The mechanism of growth modulation performed by galectin-1 might be through its association with the oncogenic protein H-Ras (Paz et al., 2001; Elad-Sfadia et al., 2004). Cell cycle arrest was demonstrated for galectin-12 and may be associated with terminal cellular differentiation (Yang et al., 2001).

Both adhesive and growth regulatory properties of galectins are of particular importance for tumour development and metastasis. Variations in glycosylation

patterns and glycan structures on the surface of tumour cells strongly affect galectin binding and functions for these cells (Dube and Bertozzi, 2005; Ohtsubo and Marth, 2006; Guo et al., 2008; Zhuo et al., 2008). Also the expression of galectins in cancer cells is often altered, either increased or lost. The detailed description of these alterations is beyond the scope of this thesis and was reviewed elsewhere (van den Brule et al., 2004; Berberat et al., 2001; Lahm et al., 2004; Takenaka et al., 2004; Huflejt and Leffler, 2004; Kageshita et al., 2002). Also a change in subcellular localization of galectins has been observed and related to cancerous phenotype (Liu and Rabinovich, 2005).

Cell differentiation is another process where galectins have a role. Changes in the expression profile of galectins were reported during the differentiation of myeloid cells (Abedin et al., 2003). Progression to neutrophil and eosinophil lineages was associated with an increase in galectin-10 transcript, while galectin-3 was upregulated during monocyte differentiation. Galectin-7 is a marker for keratinocyte differentiation (Saussez and Kiss, 2006) and appears at the time of epidermal stratification. As dysregulated differentiation is a feature of cancer, the altered expression of galectins seen in many cancers, may be linked to disruption of normal cell differentiation.

Pro-apoptotic properties were demonstrated for the majority of human galectins including: galectin-1 (Perillo et al., 1997), galectin-2 (Sturm et al., 2004), galectin-3 (Fukumori et al., 2003), galectin-7 (Kuwabara et al., 2002), galectin-9 (Kageshita et al., 2002; Wada et al., 1997) and galectin-12 (Hotta et al., 2001). The important role for galectins in apoptosis of immune cells is discussed in more detail below.

1.1.4 Role of galectins in immunomodulation

A very important feature of galectin family members is their immunomodulatory properties and indeed this has been the central focus of this thesis. They act as cytokines, chemokines, growth factors as well as delivering cell death or survival signals. Since a more detailed description of the functions of galectin-3 and -9 is presented in later sections, this passage will primarily focus on other members of the galectin family.

1.1.4.1 Galectins and adaptive immunity

Galectins were found to have a profound effect on T cell biology. The best studied example in this area is galectin-1, an important inducer of T cell apoptosis. Galectin-1 is expressed on thymic epithelial cells and takes part in clonal deletion of thymocytes during development (Perillo et al., 1997). This process promotes central tolerance by eliminating autoreactive lymphocytes. Galectin-1 contributes also to the termination of immune responses by inducing programmed cell death in activated but not resting T cells (Perillo et al., 1997; Ilarregui et al., 2005). T cell surface molecules bound by galectin-1 are CD45, CD43, CD3, CD7 (Pace et al., 2000; Pace et al., 1999; Elola et al., 2005). Ligation of these molecules by galectin-1 initiates various signalling cascades including death receptor and mitochondrial pathways and caspase-independent mechanisms (Matarrese et al., 2005; Brandt et al., 2008; Hahn et al., 2004). Further proof for a role of galectins in T cell apoptosis came from a study by Toscano et al. which demonstrated that susceptibility of Th1 but not Th2 cells to galectin-1 induced apoptosis depends on differential glycosylation of surface molecules in these cell subpopulations (Toscano et al., 2007). Due to the

immunosuppressive role of galectin-1 its overproduction might be used by certain tumour cells as a way to escape immune surveillance (Liu and Rabinovich, 2005). In normal conditions high expression of galectin-1 was observed at immune-privileged sites such as cornea, placenta, testis or brain (Rabinovich et al., 2000) and probably serves as a mechanism preventing activated T cell infiltration by induction of apoptosis.

Apart from its apoptotic function, galectin-1 can modulate signalling from T cell receptors (TCR) (Chung et al., 2000; Liu et al., 2009) and TCR-directed selection (Liu et al., 2008). Cytokine production may also be influenced by galectin-1, specifically suppression of IL-2 and IFNγ secretion from activated T cells (Vespa et al., 1999; Rabinovich et al., 1999; Camby et al., 2006).

With regard to other galectins, which overall have received less attention than galectins 1 and 3, a pro-apoptotic effect towards T cells was also shown for galectins -2 and –9 (Sturm et al., 2004; Wada et al., 1997; Fukumori et al., 2003). Interestingly, different surface molecules seem to be utilized by various galectins to induce cell death, galectin-3 cross-links CD29 and CD7 (Fukumori et al., 2003) and galectin-9 binds to TIM-3 (Zhu et al., 2005). An interesting recent finding is a co-stimulatory function of galectin-8 for T lymphocytes in the spleen which can induce both proliferation and cytokine production by these cells (Tribulatti et al., 2009). Galectin-10 has also been identified as a marker for regulatory (CD4+CD25+Foxp3+) T cells maintaining their anergic state (Kubach et al., 2007).

1.1.4.2 Galectins and innate immunity

The impact of galectins on immune cells is not limited to T cells and includes modulation of chemotaxis. Galectin-3 is a strong chemoattractant towards monocytes and macrophages (Sano et al., 2000) and galectin-9 acts on eosinophils (Matsumoto et al., 1998). Activation of cells of the innate immune system (neutrophils or macrophages) is another of their functions. Release of toxic oxidative metabolites (oxidative burst) in primed neutrophils that have transmigrated through the endothelium can be induced by both galectin-1 and -3 (Almkvist et al., 2002; Karlsson et al., 1998). Furthermore, galectin-3 enhances phagocytic activity of macrophages and their production of inflammatory mediators (Sano et al., 2003) and opsonizes apoptotic neutrophils to facilitate their clearance (Karlsson et al., 2009). Galectin-1 on the other hand modulates expression of receptors for the Fc portion of immmunoglobulins (FcγR) which mediate phagocytosis and inhibit the expression of major histocompatibility complex (MHC) II molecules which enable macrophages to present antigens to T cells (Barrionuevo et al., 2007). In general, these two galectins have opposing pro- and anti-inflammatory roles. For example galectin-3 causes activation of mast cells, whereas galectin-1 prevents their degranulation (Chen et al., 2006; Rabinovich et al., 2000). Galectin-1 inhibits neutrophil chemotaxis and transendothelial migration (La et al., 2003) while galectins -3 or -8 stimulate this process (Sato et al., 2002; Nishi et al., 2003). Some role in pathogen recognition was also proposed for galectins as they can bind to non-host carbohydrate structures on the surface of various microorganisms (Mey et al., 1996; Sato and Nieminen, 2004; Vasta, 2009).

Another anti-inflammatory function of galectins is their contribution to the clearance of activated immune cells. Treatment with galectin-1 induced exposure of phosphatidylserine on the surface of activated neutrophils preparing them for recognition by phagocytes (Dias-Baruffi et al., 2003). Similar effect on neutrophils but not T cells was reported for galectin-2 and -4 (Stowell et al., 2007).

1.1.4.3 Galectins and immune pathology

All these functions of galectins have implications for immune related disease, including chronic inflammation, autoimmunity, infection and cancer. Several in vivo models have shown a role for galectins in chronic inflammation (Rubinstein et al., 2004). Anti-inflammatory effects of galectin-1 were documented in collagen-induced arthritis (CIA) where administration of this protein suppressed development of the disease (Rabinovich et al., 1999). Galectin-1 also prevented experimental autoimmune encephalomyelitis (EAE) (Offner et al., 1990), liver injury in concanavalin A-induced hepatitis (Santucci et al., 2000) and suppressed colitis (Santucci et al., 2003). A beneficial effect of galectin-1 was found in graft-versus-host disease (GvHD) (Baum et al., 2003).

On the other hand, a role for galectin-3 in development of airway inflammation has been demonstrated in a murine model of asthma (Zuberi et al., 2004; Lopez et al., 2006). Galectin-3 knock-out animals had attenuated inflammatory responses (Hsu et al., 2000). Other studies suggest that it may also play a role in the pathogenesis of autoimmune disease (Dumic et al., 2006). Indeed, the severity of autoimmune encephalomyelitis in galectin-3 knockout animals was markedly reduced (Jiang et

al., 2009). Due to the proposed role for galectin-3 in chronic inflammation this galectin will now be discussed in more detail.

1.1.5 Galectin-3

Galectin-3, described earlier under a variety of names (Mac-2, CBP-35, HL-29, LBP) (Dumic et al., 2006), has a unique structure among the galectins. The atypical N-terminal domain responsible for multimer forming can be selectively cleaved by matrix metalloproteinases (MMP-2, MMP-9) (Ochieng et al., 1998a). Such modification increases the affinity of the CRD domain but reduces self-aggregation and thus impacts upon certain biological properties.

A characteristic anti-death sequence motif similar to BH1 domain found in Bcl-2 family proteins is present in galecitn-3 within the carbohydrate-recognition domain (Akahani et al., 1997). This explains the association with Bcl-2 proteins and the mechanism by which galectin-3 exerts its anti-apoptotic effect (Yang et al., 1996). Apart from Bcl-2, other intracellular molecules identified as binding partners for galectin-3 include components of signalling pathways: K-Ras, AIP1 or synexin in the cytoplasm and Gemin4, carbohydrate binding protein 70 (CBP70) and β-catenin in the nucleus (Dumic et al., 2006; Shimura et al., 2004). Transport between these two compartments through the nuclear pores depends on phosphorylation of galectin (Takenaka et al., 2004) and is mediated by importins (Nakahara et al., 2006).

Based on the observation that galectin-3 associates with ribonucleoproteins (RNP) of the nuclear matrix and the RNA cleavage capacity assayed in a cell-free system, a role for galectin-3 in pre-mRNA splicing was put forward (Wang et al., 1995; Dagher et al., 1995).

In fibroblasts the localization of galecitn-3 in subcellular compartments varies depending on the proliferative status of the cell. In quiescent cells it was found mostly in the cytoplasm while in dividing cells it translocated to the nucleus (Liu et al., 2002). This growth-related localization as well as increased expression in proliferating cells implies participation of galectin-3 in regulation of cell proliferation. Positive growth regulatory functions on various cells were described following transfection of galectin-3 into T cells (Joo et al., 2001) and breast cancer cells (Honjo et al., 2001). It was also implicated in control of the cell cycle through G1 or G2/M arrest (Lin et al., 2000; Kim et al., 1999b). While intracellular galectin-3 protects from apoptosis induced by Fas ligation or a broad spectrum protein kinase inhibitor staurosporine (Yang et al., 1996) the same protein located exogenously may promote cell death, as exemplified by apoptotic effect on T cells (Fukumori et al., 2003).

However, most functions described for this galectin seem to amplify rather than shut down an inflammatory process and to support this conclusion, elevated levels of serum galectin-3 have been reported in various inflammatory diseases, for example rheumatoid arthritis (Ohshima et al., 2003) and Behcets disease (Lee et al., 2007). Its pro-inflammatory role is indicated by a variety of effects on immune processes. For example, galectin-3 facilitates the influx of immune cells into tissues. Interactions of circulating leukocytes with endothelium are primarily mediated by selectins and integrins but galectins may further strengthen the adherence of these cells to vessel walls. It was demonstrated that galectin-3 promotes both neutrophil extravasation (Sato et al., 2002) and adhesion to laminin (Kuwabara and Liu, 1996). Furthermore, in galectin-3 knockout mice the recruitment of phagocytic cells in response to

inflammatory agent was severely impaired (Colnot et al., 1998). Through binding to cell surface glycoconjugates galectin-3 also activates various immune cells. Respiratory burst in both neutrophils and monocytes is induced in the presence of galectin-3 (Karlsson et al., 1998). It also increases neutrophil bacteriostatic and phagocytic functions (Farnworth et al., 2008). Another recent finding revealed that galectin-3 can reverse the desensitized state of neutrophils caused by chemotactic peptide fMLP (formyl-Met.Leu-Phe peptide) (Forsman et al., 2008). Cross-linking of IgE receptors, particularly on basophils, triggers degranulation and cytotoxicity towards intracellular parasites (Zuberi et al., 1994).

Macrophages are also recruited to sites of inflammation by galectin-3. It was demonstrated that galectin-3 exhibits more potent chemotactic properties for monocytes and macrophages than the classical chemokine monocyte chemoattractant protein-1 (MCP-1/CCL2) (Sano et al., 2000). In contrast to MCP-1 whose receptor is present on monocytes but not macrophages, galectin-3 binds to different surface molecules and acts on both cell types. Galectin-3 binding enhances phagocytosis by these cells (Sano et al., 2003) and production of cytokines (IL-1) (Jeng et al., 1994; Almkvist and Karlsson, 2004). Through binding to CD98 receptor galectin-3 regulates alternative (i.e. IL-4-induced) macrophage activation (Mackinnon et al., 2008). Monocyte chemoattraction and induction of various chemokines from macrophages was implicated in vascular inflammation of atherosclerosis (Papaspyridonos et al., 2008). In addition, galectin-3 was shown to induce mediator release by mast cells (Chen et al., 2006) and therefore may play a role in allergic reactions. Galectin-3 also affects angiogenesis as it can attract endothelial cells (Nangia-Makker et al., 2000).

1.1.6 Galectin-9

Galectin-9 belongs to the tandem-repeat class of galectins i.e. it consists of two carbohydrate-recognition domains (CRDs) connected by a link peptide. Frame-shift mutations leading to a truncated product with only one functional CRD were found in the coding sequence of galectin-9 in some cancer cells (Lahm et al., 2000). Three isoforms that differ in the length of this linker regions have been identified (Chabot et al., 2002). These isoforms are thought to arise from alternative splicing of the original transcript. The full-length protein may also be post-transcriptionally cleaved by enzymes such as thrombin (Nishi et al., 2006).

Galectin-9, known also as ecalectin, was first described as a selective chemoattractant for eosinophils (Matsumoto et al., 1998). New data accumulated since the first discoveries indicate that it has much broader functions. Galectin-9 is expressed in: endothelial cells, macrophages, T cells (Jurkat) (Spitzenberger et al., 2001; Chabot et al., 2002). It can be localized both intracellularly and, unusually for a galectin, on a cell surface. Release from the cell surface to a soluble form requires action of certain matrix metalloproteinases (Hirashima et al., 2004).

In parallel with identification as an eosinophil chemoattractant, galectin-9 was described as a urate transporter in the kidney (Lipkowitz et al., 2004). It functions as a voltage-sensitive channel fused into the lipid bilayer and mediates transport of urate. This product of purine metobolism is elevated in serum of individuals with renal dysfuctions and associated with development of conditions such as gout. Mediation of cell adhesion and aggregation was also demonstrated for galectin-9 and it has been implicated in the metastatic potential of tumour cells (Kageshita et al., 2002; Irie et al., 2005).

Of relevance to this thesis, galectin-9 also serves a variety of immunological functions. It regulates interactions between thymic epithelial cells and thymocytes and promotes apoptosis of immature thymocytes (Wada et al., 1997). Similar to galectin-1 it induces cell death of mature activated T cells through caspase and calpain-dependent pathways (Kashio et al., 2003). The surface receptor that is crucial for galectin-9 function is TIM3 (T cell immunoglobulin- and mucin domain-containing molecule) characteristic for fully differentiated T helper type 1 lymphocytes (Zhu et al., 2005; van de Weyer et al., 2006). Dysregulation of this pathway was documented in autoimmune diseases such as diabetes or multiple sclerosis (Koguchi et al., 2006; Hastings et al., 2007). Activation of the galectin-9/TIM3 pathway was also shown to be important in graft rejection (Naka et al., 2009). In addition to pro-apoptotic activity, galectin-9 induces differentiation of naïve T cells into a regulatory phenotype while inhibiting their development into Th17 cells (Seki et al., 2008) and stimulates maturation of dendritic cells with production of Th1-type cytokines (Dai et al., 2005).

With the emerging role for galectins in immune modulation and inflammatory diseases the present study examined galectins in the specific context of rheumatoid arthritis.

1.2 Rheumatoid arthritis

1.2.1 General information

Rheumatoid arthritis (RA) is a chronic, inflammatory, systemic disease characterised by inflammation and destruction of peripheral joints which leads to deformity and

disability. It primarily affects small joints of the hands and feet but can progress involving other joints in a symmetrical fashion (knee, hip, elbow, shoulder; (Zvaifler, 2006). RA usually begins over weeks or months with pain, stiffness and swelling of the joints. In more advanced disease extra-articular manifestations may be present including vasculitis, scleritis, pericarditis, and subcutaneous and pulmonary nodule formation (Young and Koduri, 2007). RA prevalence in the adult population worldwide is estimated at around 1%. The condition is more common in women then in men (ratio 3:1) and the peak onset of the disease is in the fifth decade of life. RA is not just a chronic condition that reduces quality of life, it decreases life expectancy by 3-10 years (Calvo-Alen and Alarcon, 2006).

The aetiology of this disease is not fully understood and several factors and pathways are implicated in its pathology. Genetic factors predisposing to the occurrence of rheumatoid arthritis include certain MHC class II alleles (HLA-DR4-related) sharing the same epitope (Gregersen et al., 1987) or the PTPN22 (protein tyrosine phosphatase) gene which confers susceptibility to various autoimmune diseases. Other candidate gene associations are PADI4 (peptidylarginine deaminase) or CTLA4 (cytotoxic T lymphocyte-associated antigen), to name but two (Gregersen et al., 2006). Overall the genetic contribution is thought to account for around 30% of individual's susceptibility to RA. Infectious agents have been implicated in the aetiology of RA (Toivanen, 2006; Rashid and Ebringer, 2007), though direct evidence of infection in affected patients is lacking. It is, however, documented that infections may trigger flares of the disease and contribute to its progression (Hyrich and Inman, 2001). Hormonal factors are also likely to modulate the susceptibility and course of arthritis as suggested by the sex imbalance in the occurrence of RA (Calvo-

Alen and Alarcon, 2006) and the fact that females experience clinical fluctuations in RA symptoms during the menstrual cycle and pregnancy (Cutolo and Lahita, 2005). Another environmental factor with an emerging role in the onset of RA is smoking (Klareskog et al., 2007; Harrison, 2002; Lundstrom et al., 2009).

Rheumatoid arthritis, as defined by the ARA criteria modified in 1987 (Arnett et al., 1988), appears as a group of heterogeneous conditions with varied clinical courses and outcomes. The disease ranges from mild non-erosive arthritis to a progressive multisystem disorder (Lee and Weinblatt, 2001). The most common features used in diagnosis are serum positivity for rheumatoid factor (RF) which is an antibody reacting with Fc portions of autologous IgG molecules and antibodies against cyclic citrullinated peptides (anti-CCP) (Nielen et al., 2004; Raza et al., 2005b).

1.2.2 Histopathology of RA

The most characteristic histological feature of the rheumatoid joint is the hypertrophy of the synovial membrane accompanied by persistent leukocyte infiltrate and increased synovial fluid volume. The synovial membrane is normally a thin (1-3 cells thick) layer which lines the non-cartilaginous surfaces of the joint cavity. Two types of cells can be distinguished in this intimal lining layer, namely macrophage-like synoviocytes (or type A) and fibroblast-like synoviocytes (or type B, referred to later as synovial fibroblasts). The sublining layer is composed mainly of extracellular matrix with scattered blood and lymphatic vessels, adipocytes and fibroblasts (Tak, 2006).

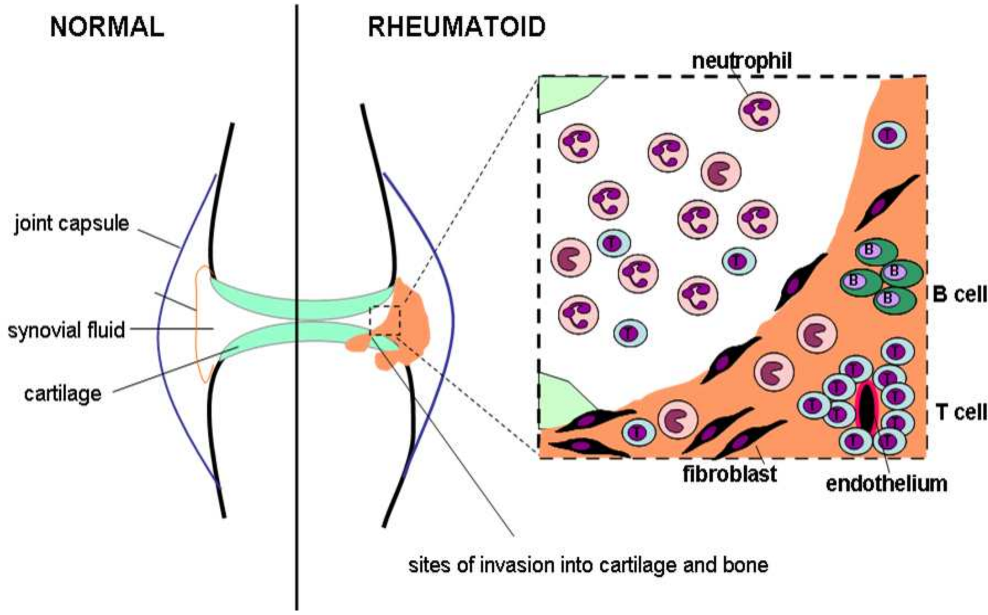


Fig. 1.3 Schematic representation of joint structure in health and disease.

Left side represents normal joint structure, right side changes that occur in rheumatoid arthritis which include pannus invading cartilage and bone, outgrowth of synovial tissue into the joint cavity and presence of infiltrate consisting of various immune cells.

In RA the synovium becomes hyperplastic, overgrows into the joint cavity and invades local articular structures along with the underlying bone. This aggressive tissue, often called pannus, is able to destroy components of the extracellular matrix via expression of degradative enzymes, including metalloproteinases, serine proteases and aggrecanases (Firestein, 2003; Rengel et al., 2007). The rheumatoid joint is also heavily infiltrated with immune cells. Neutrophils are present in large numbers in the synovial fluid (Edwards and Hallett, 1997). Monocytes, plasma cells, T and B lymphocytes increase the cellularity of the sublining layer (Fig.1.3).

Follicular dendritic cells as well as mast cells can also be found in the rheumatoid synovium. There is marked formation of new blood vessels (neoangiogenesis) which provide oxygen and nutrients to support the expanded tissue (Paleolog, 2002). Of interest, microvessels in rheumatoid synovial tissue closely resemble those found in the lymph nodes (Szekanecz and Koch, 2000). This abnormal vascular structure may facilitate pathological homing and transmigration of leukocytes into the synovium. Moreover, the expanded endothelial cell themselves are capable of cytokine production and leukocyte recruitment (Szekanecz and Koch, 2000) and therefore contribute to persistence of disease.

As mentioned previously, RA is emerging as a heterogenous disease and this is also reflected in its histological picture (Weyand and Goronzy, 2003). Distinct types of leukocyte synovial infiltrate have been described (Takemura et al., 2001). In some cases infiltrating T and B lymphocytes may acquire a high degree of cellular organization forming follicles with germinal centre (GC) reactions, i.e. similar to those arising in secondary lymphoid tissues during antigen-specific responses. Indeed these lymphoid microstructures in the synovium resemble those found in peripheral lymph nodes and have been named tertiary lymphoid tissue. In another pattern of lymphocytic infiltrate T cells and B cells form aggregates but without follicular dendritic cells inside and therefore lacking germinal centre reactions. The third subtype is characterized by a diffuse distribution of lymphocytes without apparent clustering. These distinct phenotypes of lymphoid architecture in the synovium are associated with differences in production of cytokines and chemokines (Klimiuk et al., 1997; Klimiuk et al., 2005) which further influences clinical

parameters and outcome. For example, the type with the highest organisation of lymphoid infiltrate with GC-like follicles correlates with an aggressive disease and was not observed in seronegative patients (i.e. patients without detectable rheumatoid factor) (Silverman, 2006). It is not established whether these histological patterns change over time or with the disease progression.

1.2.3 Components of the inflammatory infiltrate

Neutrophils

Neutrophils are part of the innate immune system and possess potent phagocytic and anti-microbial functions. They are normally short-lived cells but their life span can be increased when recruited to the sites of inflammation. In the synovial fluid of patients with active rheumatoid arthritis neutrophils constitute the most abundant cell type (Edwards and Hallett, 1997). Interestingly, they are scarce in the synovial tissue itself with the exception of the cartilage-pannus junction which is the primary site of erosion (Tak et al., 1997). The relative absence of neutrophils from the synovium may result from specific expression of integrin receptors which would promote migration of the cells through the tissue (Gao and Issekutz, 1997) rather than retain them inside. In order for neutrophils to migrate into sites of inflammation they have to be previously activated. Such activation may occur via receptors for constant region of immunoglobulins (Fc receptors) (Edwards et al., 1997) and may be triggered by immune complexes (Coxon et al., 2001). In RA patients immune complexes (formed by antigens cross-linked by multivalent antibodies) were found both in the joint deposited on cartilage as well as in the circulation (Mageed et al., 1991). It was also shown that cytokines present in the rheumatoid joint enhance neutrophil activation by immune complexes (Fossati et al., 2002). Once in the joint

neutrophils contribute to the local production of proinflammatory cytokines such as tumour necrosis factor (TNFα), interleukin-1 (IL-1), IL-6 and chemokines: CXCL8 (IL-8), CCL3 (MIP-1α), CCL4 (macrophage inhibitory protein, MIP-1β), CXCL1 (growth regulated oncogene, GROα) (Cassatella et al., 1997; Scapini et al., 2000). In addition, neutrophils' release of reactive oxygen intermediates and granule constituents can create a toxic microenvironment in the joint space. These polymorphonuclear leukocytes contain a variety of potent degradative enzymes in their intracellular granules (procollagenase, elastase, gelatinase) which are capable of cartilage destruction (Hilbert et al., 2002; Velvart and Fehr, 1987). On the other hand, reactive oxidants may cause damage to various biological molecules. For example, degradation of proteoglycans and hyaluronate by hypochloric acid was shown to decrease synovial fluid viscosity and hence its lubricative properties (Schiller et al., 1996).

A critical role for neutrophils was shown in animal models of autoantibody-mediated arthritis (Wipke and Allen, 2001) where depletion of these cells prevented the onset of joint inflammation.

Macrophages

Another cell type that contributes considerably to inflammation and joint damage in RA is the macrophage. Macrophage population in the inflamed synovial membrane is significantly expanded and their numbers correlate with radiological progression of joint destruction (Mulherin et al., 1996). Moreover, they show an activated phenotype as defined by expression of MHC class II molecules and production of inflammatory mediators (Kinne et al., 2007). Two subpopulations of macrophages

have been described based on differential expression of proinflammatory and regulatory cytokines (Mills et al., 2000). An important function for macrophages is the clearance of apoptotic cells and immune complexes (Blom et al., 2003). The expression of receptors for the latter (FcγRIIIa) seems to be unique for synovial macrophages in RA. Macrophages take part in antigen presentation and activation of T cells (McInnes et al., 2000; Brennan and Foey, 2002). Interactions of these two cells types has a major role in induction of TNFα (Brennan and Foey, 2002). As already mentioned, in the synovial membrane macrophages are the major source of TNFα. However, they also generate a large array of other mediators including interleukins: 1, 6, 15, GM-CSF (granulocyte-macrophage colony stimulating factor), CXCL8, CCL2, CCL3, proinflammatory prostaglandins and leukotrienes, to name but a few (Szekanecz and Koch, 2007). These mediators are involved in crucial pathological processes such as recruitment of other inflammatory cells, activation of synovial fibroblasts or development of new blood vessels. Macrophages produce also anti-inflammatory cytokine IL-10 and IL-1receptor antagonist (IL-1Ra) which normally take part in termination of an inflammatory response. The perpetuation of the disease depends on the balance between these signals in RA synovium (Miossec, 2004).

Although the production of matrix metalloproteinases (MMP-9, MMP-12) elaborated by a macrophage population may have little effect on degradation of cartilage matrix components, these cells are regarded as important amplifiers of the destruction mediated by fibroblasts (Kinne et al., 2006). Indeed contact of these two cell types enhances the production of mediators such as IL-6, IL-8 or GM-CSF (Chomarat et al., 1995). Another possibility of macrophages' contribution to joint destruction is

their differentiation, under the influence of receptor activator of NFκB ligand (RANKL), into osteoclasts responsible for bone resorption (Danks et al., 2002).

T cells

T cells are present in the synovium in large numbers particularly in areas surrounding blood vessels. It is proposed that the transition of T cells to the synovium may occur in an antigen-independent way and be triggered by non-specific cytokine stimulation (Zhang et al., 2005). Synovial T cells are distinct in many respects from peripheral blood T cells. They display higher CD4+/CD8+ ratio (Tak, 2006) and consist mostly of memory cell as characterised by the expression of CD45RO molecule (Potocnik et al., 1990). They express activation markers such as HLA-DR, CD27, VLA-1 (very late antigen), CD69, CD28 and CD40 ligand (CD40L) (Kinne et al., 1997) but show little or no proliferation in this microenvironment (Salmon et al., 1997). Another alteration observed in RA patients is the restricted repertoire of T cell receptors in resting cells (Wagner et al., 1998; Goronzy et al., 1998). This reduced diversity in TCR repertoire is likely to result from premature senescence of these cells (Goronzy and Weyand, 2003) Signalling from antigen T cell receptors may also be defective (Maurice et al., 1997) although this has recently been questioned as an ex vivo effect (Abreu et al., 2009). The fact that RA in many cases is associated with certain major histocompatibility complex (MHC II) haplotypes that present antigens to T cells led to the hypothesis of a central role of T cells in RA pathogenesis. However, no definite arthritogenic peptide has been found although few candidates such as type II collagen, citrullinated peptides or heat shock proteins can potentially bind to the 'shared epitope' (Fox, 2009; Kinne et al., 1997). Other autoantigens proposed for RA

pathology are cartilage link protein, proteoglycan, cartilage glycoprotein gp39 (de Jong H. et al., 2009; Kotzin et al., 2000; Tsark et al., 2002).

Most T cells in the rheumatoid joint are of the type 1 helper cells (Th1) (Yamada et al., 2007; van Amelsfort et al., 2004). T cells with regulatory functions (CD4+CD25+Foxp3+) also seem to be present in RA synovial fluid but are not fully functional (van Amelsfort et al., 2004). Remarkably, only small amounts of T cell-derived cytokines such as IL-2, IL-4 or IFNγ were detected in synovial fluid from established disease indicating low T cell activity (Firestein and Zvaifler, 2002; Feldmann et al., 1996). However, for the perpetuation of an inflammatory process T lymphocytes are of crucial importance. Their interaction with synovial macrophages drives TNFα production (Brennan and Foey, 2002) and fibroblasts are stimulated to secrete IL-6, IL-8 or prostaglandin E (PGE_2) (Yamamura et al., 2001). A factor significantly contributing to synovial activation is IL-17 produced by a subset of T cells named Th17 (Chabaud et al., 1999; Toh and Miossec, 2007; Stamp et al., 2004). Particularly high levels of this interleukin were found in RA synovial fluid (Ziolkowska et al., 2000; Kotake et al., 1999). T cell are also capable of activating osteoclasts via expression of RANK-ligand (also known as osteoclast differentiation factor, ODF) (Goldring and Gravallese, 2000).

The importance of T cells in the initiation of rheumatic disease is a matter of debate in the literature as some animal models show T cell independence in the early stages (Firestein and Zvaifler, 2002). Also T cell-directed therapies showed little success although inhibition of co-stimulation by CTLA4-Ig (cytotoxic T lymphocyte antigen-4 bound to immunoglobulin heavy chain) proved to be a more promising approach (Buch et al., 2008; Smolen et al., 2007).

B cells

B cells are found in the synovium in lower numbers than T cells or macrophages, however they have important functions in the pathogenesis of disease. They can proliferate locally in the synovium, particularly within the follicle-like structures and mature into plasma cells. These terminally differentiated cells synthesize and secrete rheumatoid factor and other autoantibodies that subsequently form immune complexes (Schroder et al., 1996; Kim et al., 1999a). It was found that B cells express on their surface lymphotoxin-α and thereby contribute to the formation of the ectopic germinal centres (Fu et al., 1998). B cells are responsible for maintenance of memory response to autoantigens. Furthermore, they have a capacity for antigen presentation to T cells and provide co-stimulatory signals for their activation (Silverman, 2006). Synthesis of chemokines that induce leukocytes infiltration and factors that sustain angiogenesis constitute an additional mechanism in the pathogenesis of synovial inflammation (Mauri and Ehrenstein, 2007). The importance of B cell was shown by improvement in disease scores following B cell-depleting therapies with an anti-CD20 antibody (Edwards et al., 2004).

Dendritic cells

Dendritic cells are the most potent antigen-presenting cells. They are found in rheumatoid synovium (Page et al., 2002) particularly within the aggregates of T and B cells and in perivascular lymphocytic areas (Pettit et al., 2000). It is generally accepted that under normal conditions dendritic cells migrate from local tissues to lymph nodes. A possible cause for their retention in the synovial tissue is the expression of ligands for lymph node-homing receptor CCR7 such as CCL19 and

CCL21 by rheumatoid synovial fibroblasts (Page et al., 2002). Dendritic cells in the synovium serve primarily as antigen-presenting cells for memory T cells as suggested by their localization in close proximity to CD4+ T cells (Tak, 2006). Follicular dendritic cells (FDCs) also play a role in the accumulation of B cells and their differentiation into plasma cells (Schroder et al., 1996b) thereby promoting humoral immune responses. Production of cytokines and chemokines (eg. IL-1β, TNFα, CCL3, CCL4) by dendritic cells is another feature regulating the inflammatory process (Foti et al., 2004). Finally, in the rheumatoid microenvironment they may transdifferentiate into osteoclasts (Rivollier et al., 2004).

Taken together this cellular inflammatory infiltrate, among other functions, drives the local cytokine production and leads to persistence of the disease processes (McInnes and Schett, 2007);Buckley 2003b). It is becoming apparent that resident synovial fibroblasts play an important role in the retention of immune cells in the inflamed joint (Huber et al., 2006). Their contribution to the perpetuation of the disease will be described in later sections.

1.3 Osteoarthritis

Osteoarthritis (OA) is another most common cause of joint dysfunction and disability. In contrast to RA, pathological changes involve mainly large, weigh-bearing joints (hips, knees). The main feature of the disease is joint space narrowing, cartilage mineralization and new bone formation (osteophytes) within the joint that impairs its function. Although the mechanisms leading to cartilage loss are not

entirely understood, biomechanical factors such as usage and trauma are believed to play a main role in its pathology (Samuels et al., 2008).

Osteoarthritis used to be regarded as a non-inflammatory disease and as such opposed to RA. However, the inflammatory component is not entirely absent and markers of inflammation such as raised levels of C-reactive protein (CRP) or hyaluronic acid are often associated with active disease (Pelletier et al., 2001). It is unclear whether the inflammatory episodes observed during the course of the disease are primary events or whether they result from other destructive changes. Moreover, at later stages of the disease, the histopathological picture of synovitis may be indistinguishable from RA (Samuels et al., 2008). Cytokines and chemokines characteristic for RA such as TNFα, IL-1 or IL-6, are also present in OA although at lower levels (Farahat et al., 1993; Smith et al., 1997a) which likely results from lower density of inflammatory infiltrate.

1.4 Fibroblasts

1.4.1 Fibroblasts and the immune system

Fibroblasts are the most important cell type constituting tissue stroma. They are responsible for extracellular matrix synthesis, degradation and rebuilding of the tissue during wound healing. However, their function is not limited to providing structural scaffold for tissues and organs. Fibroblasts may also be considered as part of the immune system (Smith et al., 1997b). They express a wide variety of receptors, adhesion molecules and immune mediators, which enable them to sense danger signals in the environment, interact with other immune cells and regulate their behaviour. For example, recruitment of immune cells is mediated by

chemoattractants such as CXCL8 (IL-8), CCL3 (MIP-1α), CCL2 (MCP-1), CCL5 (RANTES) (Brouty-Boye et al., 2000; Kontoyiannis and Kollias, 2000). As well as initiating inflammation by recruiting leukocytes to the site of tissue injury, fibroblasts may provide signals for retention, differentiation, survival and exit to the infiltrating cells when their presence is no longer required (Parsonage et al., 2005; Smith et al., 1997b). Crucial molecules that support leukocyte survival are CXCL-12 (stromal-derived factor-1, SDF-1), CXCL-13 (B cell-attracting chemokine-1, BCA-1), CCL-21 (Bradfield et al., 2003; Parsonage et al., 2005; Buckley, 2003b), type 1 interferons (Pilling et al., 1999; Wang et al., 2003) and co-stimulation is provided by CD40 or CD106 (Buckley et al., 2004a; Brouty-Boye et al., 2000). At the end of an inflammatory response effector cells have to be cleared from the affected site to allow it to re-establish its normal integrity and function (Buckley, 2003a). It is plausible to hypothesise that by withdrawing their survival signals fibroblasts would play a pivotal role in the resolution phase. Fibroblasts also have the capacity to produce anti-inflammatory mediators limiting the acute inflammatory response such as IL-10, TGFβ (transforming growth factor), soluble TNF receptor (Ritchlin, 2000).

As described above fibroblasts play an important role during the inflammatory reaction as well as in its resolution, therefore it seems likely that, any alterations in their behaviour may lead to persistence of inflammatory state and pathology. The consequences of disordered fibroblast behaviour are clearly seen in chronic diseases such as rheumatoid arthritis.

1.4.2 Fibroblast diversity

Fibroblasts from different anatomical sites have distinctive features depending on their site of origin (Parsonage et al., 2005). These differences appear remarkably stable and are likely reflect variable demands of certain tissue types. Global transcriptome analysis showed that fibroblasts can be clustered into groups defined by anatomical site (Chang et al., 2002; Rinn et al., 2006). Moreover, even within one tissue (e.g. lung or spleen) fibroblasts were found to be heterogeneous in surface marker expression, matrix protein production and proliferation (Borrello and Phipps, 1996; Fries et al., 1994). Several studies of fibroblast transcriptomes have revealed distinct expression patterns of cytokines and chemokines and variable levels of co-stimulatory molecules (Buckley et al., 2004; Buckley, 2003c; Parsonage et al., 2003; Chang et al., 2002; Brouty-Boye et al., 2000) Such properties largely affect immunoregulatory functions of fibroblasts and therefore tissue-specificity of immune responses. Unlike other cell types, for example endothelial cells, which revert to a common phenotype when grown in vitro (Garlanda and Dejana, 1997), the tissue specific characteristic phenotypes of fibroblast are retained even when cells are cultured for several passages (Smith et al., 1997b; Chang et al., 2002) This implies existence of an imprinted ‘memory’ of their tissue identity.

The capacity of fibroblasts to modulate inflammation may also be influenced by external stimuli. Treatment with inflammatory cytokines changed the pattern of gene expression in fibroblasts of different origin (Parsonage et al., 2003). For example, TNF-α induced production of IL-6, IL-8 and CCL2 (MCP-1) in synovial but not skin fibroblasts. Conversely, skin fibroblasts responded in a similar way upon stimulation with a Th2-type cytokine, IL-4. This recent evidence suggests that differences in

stromal cell phenotype and behaviour may explain a predilection of chronic inflammatory reactions for certain sites within the body (Filer et al., 2006b).

1.5 Synovial fibroblasts in rheumatoid arthritis

Apart from the inflammatory infiltrate present in the synovium, the predominant feature of active RA is an increase in synovial fibroblast numbers. The initial events underlying this process have not been defined. It is interesting to note that fibroblast tissue expansion may occur even before chronic inflammation becomes clinically evident and may therefore represent a rational therapeutic target in early arthritis (Ospelt et al., 2004b).

1.5.1 Activation of synovial fibroblasts

Rheumatoid synovial fibroblasts show increased metabolic activity and a characteristic activated phenotype, reflected by changes to the normal spindle-shaped cytoskeleton, dense rough endoplasmic reticulum (RER) and large nucleus with prominent nucleoli indicating both active RNA metabolism and protein production (Muller-Ladner and Gay, 2006). Indeed a constitutive overproduction of matrix proteins and various cytokines by rheumatoid synovial fibroblasts has been noted (Pap et al., 2006; Bucala et al., 1991). Inflammatory cytokines secreted by fibroblasts in the synovium include IL-1β, IL-6, IL-7, IL-15, IL-16, lymphotoxin β (LTβ), type 1 IFN, or GM-CSF, whereas chemokines among others include CCL3 (MIP-1a), CCL2 (MCP-1) and CCL5 (RANTES) (McInnes and Schett, 2007; Lally et al., 2005; Ritchlin, 2000). All these mediators display potent abilities to attract and activate immune cells. Fibroblasts in the inflamed synovium are also targeted by stimuli

derived from infiltrating leukocytes such as IL-1β, TNF-α or IL-17 (McInnes and Schett, 2007) and this inflammatory microenvironment further activates them and perpetuates the disease process. In addition to having a role in the crosstalk between fibroblasts and other cell types in the synovium these cytokines provide constant autocrine stimulation and survival signals for synovial fibroblasts.

Cell-cell interactions with recruited immune cells are facilitated by up-regulated adhesion molecules. For example a member of immunoglobulin family, CD106 (vascular cell adhesion molecule-1, VCAM-1) mediates cell contact with B cells (Bradfield et al., 2003; Burger et al., 2001). More importantly adhesion molecules enable strong adhesion of fibroblasts to extracellular matrix. This attachment to cartilage and bone is a characteristic feature of RA synovial fibroblasts and distinguishes them from other forms of arthritides (Pap et al., 2006). Interactions allowing for attachment to components of cartilage such as collagens and cartilage oligomeric matrix protein (COMP) are mediated mainly by integrins, subunits α3-5 (very late antigen, VLA 3-5) and β1 (Huber et al., 2006; Rinaldi et al., 1997). Upon adhesion integrins activate signalling pathways within the cell regulating early cell cycle genes such as c-fos and c-myc and activate expression of matrix metalloproteinases (Schwartz, 1997; Sarkissian and Lafyatis, 1999). Interesting findings in the biology of rheumatoid synovial fibroblast came from studies in severe combined immunodeficient (SCID) mice (Muller-Ladner et al., 1996). This animal model displays severe defects in both cellular and humoral immune responses which make it unable to reject implants. Co-implantation of synovial fibroblasts from RA patients with normal human cartilage into SCID mouse revealed that invasion of RA

fibroblasts can occur even in the absence of inflammatory cells or other mediators. Fibroblasts from skin or osteoarthritic synovium did not show such properties.

Invasion into the cartilage proves the destructive potential of rheumatoid fibroblasts. This process is mediated by matrix degrading enzymes from various families: matrix metalloproteinases (MMPs), cathepsins and activated plasmin system. MMPs expressed by synovial fibroblasts include collagenases (MMP-1, MMP-13), stromelysin (MMP-3) and gelatinases (MMP-2, MMP-9) as well as some membrane bound forms (Pap et al., 2000; Konttinen et al., 1999). Apart from their role in tissue remodelling, matrix metalloproteinases are also involved in regulation of cytokines or other MMPs i.e. their activation from precursor forms by cleavage (Ra and Parks, 2007).

Joint destruction can also occur via proteolytic enzymes called cathepsins. Members of this family of cysteine proteases which are up-regulated in RA include cathepsins B, D, L or K (Keyszer et al., 1998; Hummel et al., 1998). They degrade collagen and proteoglycans and have also been linked to activation of proto-oncogenes. Activation of plasminogen system provides yet another route for matrix degradation. RA synovial fibroblasts synthesize high amounts of urokinase-plasminogen activator (u-PA) and the resulting active serine protease plasmin exerts direct proteolytic effect both on matrix components and pro-MMPs (Muller-Ladner and Gay, 2006). Invasiveness of rheumatoid fibroblasts varies among individuals and correlates with the rate of joint erosions (Tolboom et al., 2005).

Angiogenesis is another hallmark of the inflamed synovial tissue (Huber et al., 2006). Synovial fibroblasts are capable of promoting this process by secreting growth factors of which the most important is vascular endothelial growth factor

(VEGF) (Ritchlin, 2000; Sone et al., 2001). VEGF expression is further enhanced by low oxygen tensions which were reported in RA joint (Gaber et al., 2005). Other fibroblast-produced mediators of vascularization include transforming growth factor beta (TGFβ), fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF), the latter two having potent proliferative activities on fibroblasts as well (Muller-Ladner and Gay, 2006). Effector molecules secreted by synovial fibroblasts include also arachidonic acid-derivative prostaglandin E_2 (PGE_2) – a potent mediator of inflammation (Alsalameh et al., 2003) or osteoclast-activating RANKL (Nakano et al., 2004).

Recently a role for conserved pattern recognition receptors (Toll-like receptors, TLRs) in the activation of synovial fibroblasts has been recognised suggesting a possible role for an innate immune system in the early events of the disease. Ligation of these receptors can induce expression of adhesion molecules in fibroblasts (eg. intracellular adhesion molecule-1, ICAM-1), matrix degrading enzymes (MMP-1, -3, -13), proinflammatory cytokines such as IL-1 and TNFα and chemokines attracting inflammatory cells to the synovium (Kyburz et al., 2003; Pierer et al., 2004; Brentano et al., 2005). A role for these receptors in the onset of synovitis is further suggested by animal models of arthritis induced by bacterial products (Liu et al., 2001).

1.5.2 Transformed phenotype of RA synovial fibroblasts

The altered behaviour of fibroblasts is, however, not a mere response to cytokines or other external stimuli. There seem to be a number of intrinsic alterations in rheumatoid synovial fibroblasts often referred to as a transformed phenotype. Even in the absence of continuous external stimulation these cells show a range of

molecular changes including alterations in expression of regulatory genes, signalling cascades as well as apoptotic pathways (Burman et al., 2005; Pap et al., 2000b). One of the constitutively overexpressed proto-oncogene is egr-1 (early response gene-1) which regulates transcription of several other genes relevant to RA pathology, for example cathepsin L (Pap et al., 2006). c-fos which forms part of AP-1 transcription complex and activates transcription of MMPs was also found to be up-regulated in RA synovial fibroblasts (Asahara et al., 1997; Benbow and Brinckerhoff, 1997). A number of other oncogenes including ras, raf, myb, myc were detected in synovial cells, particularly those attaching to cartilage and bone (Pap et al., 2006) which further proves their relevance for the aggressive behaviour (Pap et al., 2004).

On the other hand tumour suppressor PTEN involved in focal adhesion regulation was absent from invasive fibroblasts (Tamura et al., 1998; Pap et al., 2000a). Another remarkable feature is the high activation of nuclear factor κB (NFκB) (Miagkov et al., 1998; Li and Makarov, 2006) which likely contributes to perpetuation of disease. This ubiquitously expressed transcription factor is normally retained in the cytoplasm by its counterpart IκB. Translocation of the released NFκB to the nucleus of RA synovial fibroblasts results in binding to promoters of its target genes such as IL-6, IL-8, CCL2, CCL5, CD40, MMP, cyclooxygenase-2 or plasminogen activator (u-PA) (Huber et al., 2006; Roman-Blas and Jimenez, 2006). TNFα and IL-1 are important activators of NFκB-dependent pathways (Fujisawa et al., 1996).

RA synovial fibroblasts display also characteristic alterations in cell death pathways and the rate of apoptosis in the activated cell population is very low (less then 3%) (Matsumoto et al., 1996). They are relatively resistant to apoptosis induced by

ligation of the surface death receptor Fas, though this depends on presence of other stimuli as well as stage of the cell cycle (Perlman et al., 2001; Kobayashi et al., 2000; Pundt et al., 2009). This phenomenon might result from an increased expression of anti-apoptotic FLIP protein (FADD-like interleukin-1 beta-converting enzyme - inhibitory protein). Inhibitors of the mitochondrial pathway of apoptosis such as Bcl-2 may also be up-regulated (Matsumoto et al., 1996). Other survival proteins that are aberrantly expressed are heat-shock protein 70 (HSP70), sentrin and sumoylated proteins (Pap et al., 2000b).

In addition to dysregulated signalling pathways some chromosomal abnormalities similar to alterations in malignant transformation were observed in synovial cells (Kinne et al., 2003). The phenotype of rheumatoid synovial fibroblasts indeed resembles that of transformed cells with an anchorage-independent growth, loss of contact inhibition and increased proliferation (Meyer et al., 2006; Li and Makarov, 2006; Ritchlin, 2000; Lafyatis et al., 1989). This phenotype is maintained in culture (Pap et al., 2000b; Zimmermann et al., 2001) and favours the hypothesis of its intrinsic cytokine-independent character. Similarly, the aggressive behaviour and invasion into cartilage occurs in the absence of external stimuli (Muller-Ladner et al., 1996).

1.5.3 Synovial fibroblasts in rheumatoid arthritis and osteoarthritis

Synovial fibroblasts from osteoarthritis patients are often used as comparators to RA fibroblasts. They lack the invasive properties of RA synovial fibroblasts and appear less activated in vivo. In culture OA fibroblast show some similarities in responses to stimuli (cytokine or chemokine expression) (Cheon et al., 2002; Pierer et al., 2004) which may reflect some generic site-determined properties. Nevertheless there are

also a number of differences in expression of genes, including proto-oncogenes, between RA and OA synovial fibroblasts (Scaife et al., 2004; Seki et al., 1998). The fact that these differences are maintained in culture (Zimmermann et al., 2001; Ritchlin et al., 1994) supports the existence of a distinctive rheumatoid phenotype characterized by stable activation.

1.5.4 Interactions of fibroblasts with immune cells in the rheumatoid joint

The first event triggering joint inflammation in human RA is not known. However, it is well established that for the development and persistence of an inflammatory state a concerted action of synovial fibroblasts and various immune cells is needed. This interplay includes secretion of soluble mediators on the one hand and direct cell-cell interactions on the other. Multiple cytokines, chemokines, growth factors and other mediators present in RA synovial tissue comprise a complex network where many factors show synergistic or complementary effects. Due to their large secretory capacity fibroblasts and macrophages are the primary producers of many inflammatory mediators. Nevertheless, other cells, for example T cells, also contribute to production of cytokines and establishment of the specific inflammatory milieu.

In the disordered synovial microenvironment fibroblasts interact with a wide variety of leukocyte subtypes promoting their survival and accumulation (Buckley et al., 2001). Moreover, such interactions are largely reciprocal and affect both cells involved. An example is the crosstalk between fibroblasts and T cells occurring via CD40/CD40L system. Engagement of the receptor by a ligand provided by activated T cells results in increased production of chemokines , adhesion molecules (ICAM-1,

VCAM-1) and proliferation of fibroblasts (Min et al., 2004; Rissoan et al., 1996). T cells can also induce production of IL-6, IL-8 or PGE_2 from synovial fibroblasts as well as matrix metalloproteinases (Yamamura et al., 2001; Schurigt et al., 2008). Fibroblasts in turn release the potent T cell chemoattractant IL-16 which facilitates influx of lymphocytes into synovial tissue (Franz et al., 1998). They also activate T cells via secreted interleukins IL-7, IL-15 or IL-18 (McInnes et al., 2000; Tran et al., 2005) therefore many positive feedback loops exist between these cells. Furthermore, fibroblasts in the synovium have the capacity to present antigens to T lymphocytes (Tran et al., 2007).

Synovial fibroblasts also promote homing, maturation and survival of B cells in the synovium. Crucial for these interaction and protection of B cells from apoptosis are the constitutively expressed chemokine CXCL12 (SDF-1) and adhesion molecule CD106 (VCAM-1) (Burger et al., 2001). Another study revealed that RA synovial fibroblasts exhibit follicular dendritic cell (FDC) activity and may play an active role in ectopic germinal centre formation (Lindhout et al., 1999).

Fibroblasts' interactions with resident or blood-derived macrophages are also of crucial importance for the inflammatory process. Chemokines released by fibroblasts such as CCL2(MCP-1) or CCL3 (MIP-1α) are major factors attracting monocytes into the synovium (Smith et al., 1997b). Macrophages in turn elaborate fibroblast-activating cytokines TNFα and IL-1β (Szekanecz and Koch, 2007). Their contribution to joint degradation processes has already been described.

In addition to stimulating leukocyte recruitment and activation in the synovium fibroblasts have a capacity to retain them at this site by providing survival signals. T cell survival is crucially dependent on the interaction with surrounding stromal cells

as the autocrine production of IL-2 is very low. It has been shown that co-culture with fibroblasts from rheumatoid synovium provides such signals for synovial T cells (Salmon et al., 1997). Soluble interferon α and β secreted by stromal cells induce up-regulation of anti-apoptotic Bcl-xL in T cells and can rescue them from cell death (Pilling et al., 1999; Gombert et al., 1996). Interestingly, synovial fibroblasts seem to be much more potent in supporting T cell survival than those derived from skin providing possible explanation for specific accumulation of leukocytes in this microenvironment (Filer, 2006).

Neutrophil survival at the site of inflammation is also increased by the interaction with stromal fibroblasts. Adhesion molecule VCAM-1 present on the surface of fibroblasts has been shown to mediate neutrophil survival (Ross et al., 2006). However, support for neutrophils seems to occur mainly by soluble factors produced both by fibroblasts and resident macrophages (e.g. IL-8, GM-CSF, TGF-β, type 1 IFN) (Kobayashi et al., 2005; Wang et al., 2003), though research in this area is relatively limited. Identification of such fibroblast-derived survival factors for neutrophils was therefore one of the subjects of this thesis.

1.6 AIMS OF THE THESIS

From the previous text it is clear that interactions between leukocytes and fibroblasts within the rheumatoid synovium play a key role in the generation and maintenance of the inflamed site, but the key factors involved in the cross-talk have not been fully characterised. Moreover the phenotype of synovial fibroblasts appears to play a specific role in maintaining the chronicity of inflammation within articulated joints. In this context the expression and role of fibroblast derived galectins in the chronic inflammation of RA was poorly understood. The influence that galectins may have on synovial fibroblasts was not defined and was another focus of the thesis.

The main questions posed at the start of the thesis were:

1. What are the neutrophil survival factors produced by cytokine activated synovial fibroblasts?
2. Do galectins play a role in neutrophil survival mediated by activated synovial fibroblasts?
3. Do fibroblasts show tissue- or disease-specific expression of galectins?
4. Do any of the differentially expressed galectins modulate function of RA synovial fibroblasts?
5. What is the mechanism of galectin-3 induced effects in fibroblasts?

2.0 MATERIALS AND METHODS

2.1 Antibodies

All antibodies used for stainings are summarized in Table 2.1.

Table 2.1

Antibody (species)	Dilution	Source
Western blot		
Primary antibodies		
Galectin-1 (rabbit)	1:1000	Peprotech
Galectin-3 (rabbit)	1:2000	Peprotech
Galectin-9 (mouse)		GalPharma
Akt/PKB (mouse)	1:1000	Transduction Lab
phospho-Akt (rabbit)	1:1000	Cell Signalling
p44/43 (ERK1/2) (rabbit)	1:1000	Cell Signalling
phospho-p44/42 (rabbit)	1:1000	Cell Signalling
p38 MAPK (rabbit)	1:1000	Santa Cruz Biotech
phospho-p38 MAPK (mouse)	1:1000	Santa Cruz Biotech
JNK1 (rabbit)	1:1000	Santa Cruz Biotech
phospho-JNK (rabbit)	1:2000	Promega
phospho-STAT1 (rabbit)	1:1000	Cell Signalling
β-actin	1:5000	Sigma
α-tubulin	1:1000	Sigma
Secondary antibodies		
Anti-rabbit-HRP (goat)	1:5000	Amersham
Anti-mouse-HRP (goat)	1:5000	Amersham
Anti-rabbit-HRP (goat)	1:10 000	Jackson Immunoresearch
Anti-mouse-HRP (goat)	1:10 000	Jackson Immunoresearch
Fluorescence microscopy		
STAT1 (mouse)	1:50	Transduction Lab
Anti-mouseIgG1-FITC (goat)	1:50	Southern Biotech
Immunhistochemistry		
Primary antibodies		
Galecin-3	Final concentration 10µg/ml	Abcam
Galectin-9	Final concentration 10µg/ml	GalPharma
Prolyl-hydroxylase β	Final concentration 10µg/ml	Acris
CD68	Final concentration 4µg/ml	Dako
Von Willebrand factor	Final concentration 10µg/ml	Abcam
Isotype controls		
Mouse IgG1	*	Dako
Rabbit IgG	*	Dako
Secondary antibodies		
Anti-mouse-biotin (goat)	1:2000	Jackson Immunoresearch
Anti-rabbit-biotin (goat)	1:5000	Jackson Immunoresearch
Anit-mouse-AP (goat)	1:20	
Anti-rabbit-AP (goat)	1:5000	

- - used at the same concentration as the primary antibody

2.2 Cell cultures

2.2.1 Fibroblast culture

All fibroblasts lines were obtained from the Rheumatology department tissue bank frozen in liquid nitrogen. Tissue samples of synovium, bone marrow and skin were collected during joint replacement surgery from patients who fulfilled the American College of Rheumatology 1987 revised criteria for rheumatoid arthritis (Arnett et al., 1988). Due to lack of detailed clinical data of the patients no correlations with parameters such as disease activity or previous therapy were made. Lung, spleen, tonsil and thyroid tissue were obtained by, respectively, bronchoscopic biopsy, splenectomy, tonsillectomy or thyroidectomy from donors not diagnosed with inflammatory disease. Collection of human tissue was approved by the local ethics committee and all patients gave consent for the use and storage of tissue for research purposes.

Cells were recovered from liquid nitrogen storage by quickly defrosting the vial at 37^0C and resuspending cells in complete fibroblast medium. After washing off the DMSO-containing freezing medium, cells were again resuspended in the medium in which they were grown. Complete fibroblast medium consisted of 87.4% RPMI 1640 (Sigma Ltd), 10% heat-inactivated FCS (Sera Laboratories International), 0.87X MEM non-essential amino acids, 0.87mM sodium orthopyruvate, 1.75mM glutamine, 87U/ml penicillin, 87µg/ml streptomycin (all from Sigma). A medium for transfection experiments consisted of Dulbecco's minimum essential medium (DMEM), 10% heat-inactivated FCS, 2 mM L-glutamine and 10 mM HEPES (all reagents provided by Gibco-Invitrogen). Cells were incubated in a humdified atmosphere at 37^0 C and 5% CO_2 and used for experiments between passages 4-8.

For passaging, mRNA isolation or nuclear extract preparation cells were trypsinized with 2X Trypsin-EDTA solution (Sigma) for 5 minutes, collected into polypropylene tubes with complete medium and centifuged for 6 min at 300g. For FACS staining cells were detached with a milder enzymatic solution Accutase (PAA Laboratiories) and washed with PBS with 1% FCS.

2.2.2 Neutrophil isolation

Neutrophils were isolated from peripheral blood of healthy volunteers who had given their informed consent. Blood was collected into Falcon™ tubes containing EDTA (3mg/ml of blood) as anti coagulant. 1 ml of 2% Dextran was added per 7 ml of blood to sediment the red blood cells. After 45 minutes leukocyte-rich plasma was collected and overlaid on to a Percoll (Sigma) gradient consisting of 79% (1.095 g/ml) Percoll (2.5 ml) and 54% (1.077 g/ml) Percoll (5 ml) in a sterile 15 ml Falcon tube and centrifuged for 20 minutes at 130g. The neutrophil enriched layer at the 79-54% interface was removed by sterile pasteur-pipette into a sterile Falcon tube. The cells were then washed in cold (4°C) PBS and spun at 270g for 8 minutes. Hypotonic lysis with cold sterile water was performed to remove any contaminating red blood cells. The number of neutrophils was counted using a haemocytometer and the cells were resuspended in RPMI 1640 medium, supplemented with 10% fetal calf serum, 100μg/ml streptomycin, 100 units/ml penicillin and 2 mM L-glutamine (all from Sigma), at a final concentration 2-4 $\times 10^6$ cells/ml. The purity of the cells was assessed by differential staining of a cytospin preparation using a commercial May-Grunwald Giemsa stain, REASTAIN Quick Diff Kit (Reagena). Only neutrophil preparations with a purity of >95% were used. Contaminating cells were mostly eosinophils.

2.3 Cell treatments

2.3.1 Cytokines

Synovial, bone marrow and skin fibroblasts were grown in fibroblasts complete medium until near confluence. The medium was replaced then with medium containing 10 ng/ml of one of the following cytokines: IL-1β, IL-6, IFNγ, TNF-α, TGFβ, IL-17 or IL-4 (20 ng/ml) and cultured for 24h before mRNA was extracted.

2.3.2 TLR ligands

The following Toll-like receptor ligands were used to stimulate synovial fibroblasts: 10 μg/ml poly(I-C) (InvivoGen), 100 ng/ml lipopolysaccharide (LPS) from Escherichia coli (List Biological Laboratories), 300 ng/ml bacterial lipoprotein (bLP, InvivoGen), 1 ng/ml recombinant interleukin-1β (R&D Systems), and 10 ng/ml tumor necrosis factor-α (R&D Systems). Cells were treated for 24h, then lysed and total RNA isolated.

2.3.3 DNA methylation inhibitor

To obtain a global demethylation of DNA in fibroblasts, cells were seeded on 6-well plates at 30-50% confluence and treated for 7 days with 0.1 or 1μM DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (5-AzaC). This inhibitor prevents the methylation of CpG islands in the promoters of genes in proliferating cells. Due to its low stability in culture, during the 7 days of treatment medium was changed every day with a fresh portion of the inhibitor. Protein cell lysates were then prepared for Western blotting.

2.3.4 Galectin-3 and inhibitors

Fibroblasts were grown on 96- or 24-well plates and stimulated with 10µg/ml galectin-3 (Peprotech). In experiments where inhibitors were used, the inhibitors were added 1h before galectin stimulation. The following signaling pathway inhibitors were used: Bay11-7085 (5µM; Alexis), LY294002 (10µM; Calbiochem), bisindolylmaleimide I (2µM; Calbiochem), Go¨6976 (10nM; Calbiochem), SB202190 (10µM; Calbiochem), R406 (10µM; supplied by David L. Simmons, Cellzome), and PD98059 (10µM; Cell Signaling). Medium containing galectin-3 with the highest concentration of DMSO found in any of the inhibitor solutions was included as a control. Cells were cultured for 24h then supernatant was taken off, spun down at 10000 rpm for 3 min. to remove any solid debris and frozen at -70^0 C for later measurement of cytokines by ELISA. Cells remaining on the plate were fixed with methanol, stained by Quick Diff and counted under a microscope to ensure the observed effect in cytokine production was not due to cell loss caused by inhibitor toxicity. Concentrations of all inhibitiors to be used were titrated in this way.

For assessment of kinase phosphorylation following galectin-3 stimulation, cells were treated with 10 µg/ml galectin-3 for 5, 15, 30 or 60 min. and cell lysates prepared for Western blotting. For STAT phosphorylation following IFNγ treatment cell lysates were prepared in the same way.

2.3.5 Fibroblast-conditioned medium production

Synovial fibroblasts were stimulated with 10 ng/ml TNF-α (Serotec) and/or 10 ng/ml IL-17A (R&D Systems) in complete fibroblast medium and incubated for 24 h.

Control fibroblasts were cultured without the addition of cytokines. Cells were then washed, resuspended in fresh medium and incubated for a further 24 hours. The conditioned media (FCM) from untreated as well as cytokine-activated fibroblasts were then harvested and stored at -70^0 C. In some experiments FCM were used immediately after collection for culturing neutrophils to avoid the effect of freeze-thawing.

2.4 Cytokine depletion and blockade studies

2.4.1 Receptor blocking on neutrophils

Freshly isolated neutrophils were incubated for 1h at 37^0 C with 2.5 µg/ml antibody against IFNβ (mouse anti-CD118, Calbiochem) or IL-6 receptor (mouse anti-IL6R, R&D Systems), with 2.5 µg/ml irrelevant IgG of the same isotype or left untreated. Cells from each condition were then plated on 96-well plates with four different treatments: medium alone, medium with 800 U/ml IFNβ (PBL Interferon Source) or 10 ng/ml IL-6 (Immunotools), respectively, fibroblast-conditioned medium (FCM) form unstimulated synovial fibroblasts and FCM from fibroblasts pre-treated with IL-17 and TNFα. Neutrophils were then cultured for 20h before the apoptosis assay was performed.

2.4.2 Cytokine depletion from FCM

To deplete fibroblast-conditioned media from cytokines protein A-conjugated agarose beads (Upstate) with immunoglobulin binding capacity were used. Beads were washed 10 times with PBS, then bound to respective antibodies for 20 min. at room temperature on a rotor mixer. The antibodies used were: anti-GM-CSF (R&D

Systems) 2 µg per depletion step, anti-TNFα (R&D Systems) 4 µg per depletion step or both together. Control beads were bound to an irrelevant IgG. Next beads were washed twice with PBS and once with RPMI and aliquoted into triplicate eppendorf tubes. Fibroblast-conditioned medium was added into a tube with antibody-bound beads or control beads and incubated for 30 min. on a rotor mixer. Beads were then spun down at 5000rpm for 5 min. and supernatant transferred into the next tube containing fresh aliquot of beads. A total of three depletion steps were performed on each FCM before they were used for neutrophil culture.

2.5 Galectin RNA expression in fibroblasts

2.5.1 Fibroblasts mRNA isolation

For analysis of gene expression cells grown on 6-well plates were washed with PBS and lysed in the wells with Lysis/binding buffer (Ambion). Total mRNA isolation was performed using RNAqueous kit (Ambion) according to the manufacturer's protocol. Any contaminating DNA was removed by using DNA*free*™ kit (Ambion) following the manufacturer's protocol. The total RNA isolated was then quantified by UV absorbance at 260nm wave length (BioPhotometer, Eppendorf).

2.5.2 cDNA synthesis

1.5 µg of the isolated RNA was immediately converted to cDNA to prevent degradation. Reverse transcription to first-strand cDNA was done with 1.25 µM oligo(dT) primer (Invitrogen), 0.25 mM dNTPs mixture and 400 units M-MLV Superscript II reverse transcriptase (Invitrogen) for 60 min. at 42°C.

cDNA samples were stored at -20°C until used for PCR amplification.

2.5.3 End-point PCR

Polymerase chain reaction was performed using 2 µl of cDNA in a 50µl reaction volume. The PCR buffer contained 1.5 mM $MgCl_2$, 0.3 mM dNTP mixture, 0.25µM of each primer and 1.75 U of Expand High Fidelity Enzyme mix containing Taq DNA polymerase and Tgo DNA polymerase (Roche). Oligonucleotide primers and PCR product size for each gene were as follows:

Table.2.2 Sequences of primers for PCR.

Gene	Forward primer	Reverse primer	Annealing temp. (°C)	Product size (base pairs)
Galectin-1	AAC CTG GAG AGT GCC TTC GA	AAC CTG GAG AGT GCC TTC GA	60	321
Galectin-2	ATG ACG GGG GAA CTT GAG GTT	TTA CGC TCA GGT AGC TCA GGT	60	358
Galectin-3	ATG GCA GAC AAT TTT TCG CTC C	ATG TCA CCA GAA ATT CCC AGT	60	719
Galectin-4	GCT CAA CGT GGG AAT GTC TGT	GAG CCC ACC TTG AAG TTG ATA	60	609
Galectin-7	ATG TCC AAC GTC CCC CAC AAG	TGA CGC GAT GAT GAG CAC CTC	65	282
Galectin-8	GTT GTC CTT AAA CAA CCT ACA G	TAA CGA CGA CAG TTC GTC CAG	60	608
Galectin-9	ACT ATT CAA GGA GGT CTC CAG	GGA TGG ACT TGG ATG GGT ACA	60	571
Galectin-10	CCA CTT GCC TGT TTC TTG AAT GAA C	TCT CCA CAC TTG CAC CAT CTT CAC	60	369
Galectin-12	CCA GCT CTA CTG TGT CCA CTC CTG AGG ATG GTT CCA GG	TAG TCT ACA ACA CTT GCC TCT GTG AGT GCA GTC CAG GC	68	308
β-actin	TTCAACTCCATCATGAA GTGTGACGTG	CTAAGTCATAGTCCGCCT AGAAGCATT	58	310

Amplification of β-actin gene was used as a control.

Thermal cycling reaction commenced with 4 min heat activation at 94°C, followed by 32-37 cycles of denaturation at 94°C for 60s, annealing at 60-65°C for 60s and extension at 72°C for 2 min. A final extension time of 10 min followed the last cycle. The amplification was performed on Px2 Thermal Cycler (Therma).

Products were separated on a 1.2 % agarose gel in Tris-Acetate-EDTA buffer and visualised using ethidium bromide staining.

2.5.4 Real-time PCR

To assess quantitative differences in gene expression real-time PCR using TaqMan® Gene Expression Assays (Applied Biosystems) was performed. Commercially available probes and primers labelled with the reporter dye FAM were used to assess galectins expression. β-actin was used as an endogenous control in general screening for galectins expression. In all other experiments 18S probe was used as a reference gene. For the PCR reaction 100ng of cDNA was taken. 10µl universal PCR master mix and 1µl of the TaqMan® Gene Expression Assay mix containing primers and probe were added and the volume was adapted with water to give a total volume of 20µl. Each reaction was performed in triplicate on a 348-well clear optical reaction plate (Applied Biosystems). Detection was performed using the ABI prism 7900HT sequence detector (Applied Biosystems). PCR cycling conditions were set to 50°C for 2 minutes, 95°C for 10 minutes and 40-45 cycles of 95°C for 15 seconds and 60°C for 1 minute. Data was collected and analysed using the ABI Prism 7900HT Sequence Detection System software, version 2.2.

Target gene expression was normalised to the endogenous control. The relative quantity of the product was calculated as follows; RQ = $2^{-\Delta Ct}$, where ΔCt is the difference in the number of cycles at the threshold line between target and control gene. The fold change of each gene expression (eg. after cytokine stimulation) was calculated as: fold change = $2^{-\Delta\Delta Ct}$, where ΔΔ Ct is the difference between ΔCt of stimulated and control sample. Where samples were not matched (i.e. in comparisons of RA vs. OA) only -ΔCt was plotted on graphs.

2.6 Protein expression and secretion

2.6.1 Immunohistochemistry

All tissue sections were obtained from Centre of Experimental Rheumatology, University Hospital Zurich (Switzerland). Sample collection was approved by local ethical committee.

Tissue fixation and preparation of paraffin blocks

Synovial tissue was fixed with 4% paraformaldehyde for up to 12h, then in 50% ethanol and embedded in paraffin by tissue processor (Shandon). Paraffin block were sectioned to 3 µm thickness on a microtome, transferred onto Superfrost slides and left to dry.

Immunohisotchemical staining

Before proceding to the staining the tissue was deparaffinized and re-hydrated by immersing the slides in xylene (2x 10min.) and graded alcohol (absolute: 2x 3min, 96%: 2 min, 80%: 2 min.) and then washed in water. Antigen retrieval was performed by pre-treatment of the tissue with 10mM citrate buffer (pH=6.0) for 30min. at 80^0 C. After washing with water the endogenous peroxidise activity was blocked by incubating the sections in 3% H_2O_2 for 15 min. Next, slides were washed with water and then with buffer1 (TBS with 0.05% Tween, ph=7.6). Blocking of non-specific immunoglobulin binding was performed by incubation with 5% goat serum and 1% BSA in buffer1 for 40min. in a humidified chamber. 100µl of diluted primary antibody or immunoglobulin of the same isotype as a negative control was applied on the slides and incubated overnight at 4^0 C in a humidified chamber. Slides were next washed for 5 min. in buffer1 and respective secondary antibody (biotinylated or enzyme-conjugated) was applied (30min. at room temperature). If

secondary antibody was biotinylated additional incubation with streptavidin-HRP complexes (Vectastain Elite ABC kit, Vector) was performed before final washing and adding a substrate for the enzyme. The substrate for horseradish peroxidise (HRP) was 3-amino-9-ethylcarbazole (AEC, Sigma) producing red colour or DAB (Vector Labs) with brown/grey-coloured product. As a substrate for alkaline phosphatase (AP) naphthol AS-MX phosphate and fast blue RR salt (Sigma) or BIICP violet kit (Vector Labs) were used. Colour reaction was stopped with water and slides mounted with glycerine/gelatine covering medium. For double staining, after developing the first reaction the whole staining procedure (starting from the blocking step) was repeated with another primary antibody. Antibodies used are summarized in Table.2.1.

2.6.2 Western blotting

To analyse cell protein content by Western blotting, cells were washed with PBS and lysed with SDS loading buffer (0.125M Tris pH 6.8, 20% glycerol, 2% SDS, 5% 2-mercaptoethanol and 25μg/ml bromophenol blue). Lysates were then heated for 10 minutes at 100°C and stored at -20°C until further use.

Depending on their size the proteins were separated on 12% or 15% SDS-PAGE gels with 5% acrylamide as a stacking gel. Molecular weight marker (Fermentas or National Diagnostics) was loaded in a separate lane on each gel. The gels were run at 40V until suitable separation was obtained. The proteins were then transferred to 0.45 micron PDVF membrane (Flowgen Limited) pre-activated shortly in methanol. Transfer was perfomed for 90 minutes at 450 mA using a wet blotting system (Biorad). The membranes were then blocked with 5% non-fat milk powder in Tris-

buffered saline (TBS; 50mM Tris pH 7.6, 150mM NaCl) with 0.1% Tween20 for 1-2h at room temperature. For analysing phosphorylated proteins the membranes were blocked with 5% BSA in 0.1% Tween-TBS. The specific primary antibodies were diluted in 0.1% Tween-TBS with 1% milk or BSA depending on the protein of interest. Antibodies used and their working dilutions are listed in Table 2.1. The membranes were incubated with an appropriate primary antibody overnight with shaking at 4^0C. The membranes were then washed 3 times for 10 minutes with 0.1% Tween-TBS and incubated with species specific secondary antibody conjugated to horseradish peroxidise for 1h at room temperature. After washing (3x 10 minutes) blots were incubated in ECL solution (Amersham Pharmacia) for about 1 minute to detect the bound antibody. The membranes were wrapped in Saran wrap and placed in film cassette boxes with an X-ray film (Kodak-x-omat LS). Time of exposure varied depending on the strength of the signal. The films were developed with an automated developing system (AGFA Curix 60). The control of equal loading was assessed after stripping the blots and re-probing them with anti-β-actin or anti-α-tubulin antibodies according to the procedure described above. Stripping was performed for 30 min. at 55^0C with agitation in 62.5mM Tris-HCl buffer (pH 6.7) with 100mM mercaptoethanol and 2% SDS.

Quantitative analysis of galectin expression was performed by scanning the developed films using Syngene gel documentation system and analysing the absorption with GeneTools image analysis software. The relative expression of a protein of interest was normalized to the loading control and represented as a percentage of the control sample value.

2.6.3 Enzyme-linked immuno-sorbent assays (ELISAs)

Levels of cytokines, chemokines, MMP-3 and galectin-3 in supernatants of cultured fibroblasts were measured by ELISA according to the manufacturer's instructions. The kits used were IL-6 OptEIA (BD Pharmingen 555220), IL-8 OptEIA (BD Pharmingen 555244), CCL5/RANTES Duoset (R&D DY278), MMP-3 Duoset (R&D DY513), galectin-3 (Bender MedSystems). The lower detection limits for IL-6 and CCL5 ELISA kits used were 15 pg/ml, for IL-8 3pg/ml, for MMP-3 30 pg/ml and for galectin-3 0.12 ng/ml.

The plates were analysed immediately using an ELISA plate reader (Molecular Devices) and Soft Max Pro software. The OD values were determined by reading the absorbance at 450nm as the primary wave length and 650nm as a reference. The values of blank wells were subtracted from the values of sample wells. Standard curve was created by plotting the mean absorbance against each concentration of the recombinant standard. The concentrations of protein in samples were calculated from the standard curve and multiplied by the dilution factor.

2.7 Transcription factor activation

2.7.1 NFκB activation - TransAM assay

To assess the activation of transcription factor NFκB (p65 subunit), nuclear extracts from galectin-3/or TNFα-stimulated fibroblasts were prepared using the Nuclear Extract Kit (ActiveMotif) according to the manufacturer's instructions. Protein content in each sample was then measured with BCA assay (Pierce) and calculated from the standard curve prepared with dilutions of BSA. Equal amounts of protein

from each sample were taken to perform the TransAM p65 assay (ActiveMotif). This assay uses microwells coated with oligonucleotides to which NFκB contained in the nuclear extract binds. Primary antibody recognizing an epitope exposed only in activated DNA-bound form of p65 subunit detects then the activated form of the transcription factor that has translocated into the nucleus. The detection was performed following manufacturer's instruction and results calculated as a fold increase compared to non-stimulated cells.

2.7.2 STAT translocation – confocal microscopy

Synovial fibroblasts were seeded onto 8-well chamber slides (BD Falcon). For STAT translocation experiments cells were serum-starved (fibroblast medium with 0.5% FCS) for 3 days with medium changed at day 2. Cells were stimulated with 100 ng/ml IFNγ (Immunotools) for 15 min then medium and plastic well chambers were removed and slides were washed with TBS for 10 min and fixed with 4% paraformaldahyde. After washing with TBS 3 times for 5 min permeabilization step was perfomed with ice-cold methanol for 10 min at -20^{0}C. Blocking buffer (10% FCS in TBS) was applied on the slides for 30 min at room temperature. Next slides were incubated with primary mouse anti-human STAT1 antibody diluted 1:50 in 2% blocking buffer for 1h in a humidified chamber. Primary antibody was omitted in negative control wells. Slides were washed twice with TBS for 10 min, secondary anti-mouse fluorochrome-conjugated antibody was applied and incubated as previously. Slides were washed twice and nuclei were counterstained by dipping slides in propidium iodide (PI) solution for 1-2 min. After final wash slides were mounted with 2.4% 1,4-diazabicyclo[2.2.2]octane (Aldrich) in glycerol pH 8.6

(Fisons Scientific). A Zeiss 510 laser-scanning confocal microscope was used to visualise staining with images captured and processed using the Zeiss LSM Image Examiner software (Zeiss).

2.8 Galectin-9 silencing

Small-interfering RNAs (siRNA) play a role in post-transcriptional gene silencing. The mechanism involves recognition and binding of short RNA sequences to target mRNA molecules which results in mRNA degradation and therefore prevents its translation into a protein. This phenomenon can be utilised to experimentally silence specific genes.

Galectin-9 siRNA used in these studies contained four different sequences targeting all isoforms. Control siRNA was a mixture of nucleotide sequences not binding any know target mRNAs.

Synovial fibroblasts were seeded on 12-well plates at a density of $5x10^4$ in a medium without antibiotics. Next day two hours before the transfection the medium was exchanged. Galectin-9 siRNA and control siRNA (Santa Cruz Biotechnology) were diluted with Opti-MEM I Reduced Serum medium (Invitrogen) and then combined with transfection agent Lipofectamine2000 (Invitrogen) prepared according to the manufacturer’s instruction. The mixture was incubated at room temperature for 20min. to form complexes before adding to the cells. The final concentration of each siRNA added to the cells was 33nM. After 24, 48 and 72h cell lysates for RNA isolation and for Western blotting were prepared. Additionally supernatants for ELISA were collected after 72h.

2.9 Functional assays

2.9.1 Neutrophil survival assays and assessment of apoptosis

Freshly isolated neutrophils were resuspended at a concentration of $2x10^6$ cells/ml. 50 µl of cell suspension was placed into 96-well flat-bottom plates and 100 µl of neutrophil medium or fibroblast-conditioned medium (FCM) was added to each well. Recombinant proteins used in neutrophil survival studies are listed in Table 2.3.

Table.2.3 Recombinant proteins used in neutrophil survival assays

Recombinant Protein	Working concentration	Source
GM-CSF	100 pg/ml	Peprotech
IFNβ	800 U/ml	Biosource International
IL-6	1-100 ng/ml	Peprotech
TNFα	1-100 ng/ml	R&D Systems
Galectin-3	0.1-10 µg/ml	Peprotech

Neutrophil apoptosis was evaluated by analyzing the loss of mitochondrial membrane potential which is an early event in programmed cell death preceding cell shrinkage and nuclear fragmentation (Zamzami et al., 1995). Cells (2×10^6/ml) were incubated with a cationic fluorochrome $DiOC_6$ (3,3'-dihexyloxacarbocyanine iodide; Molecular Probes) at a concentration of 40 nM for 30 min at 37°C. $DiOC_6$ is a positively charged molecule that permeates through the plasma membrane and accumulates in mitochondria due to their large negative membrane potential. This membrane potential is lost in cells undergoing apoptosis and the fluorescence signal from $DiOC_6$ decreases. Therefore healthy cells can be distinguished by high $DiOC_6$ staining whereas $DiOC_6$ low cells are apoptotic. After incubation with the dye the cells were washed twice with PBS and analyzed by flow cytometry. Flow cytometry

was performed using a fixed volume (20 µl) counts protocol on a Beckman Coulter Epics XL flow cytometer. Data was analyzed with the Win MDI version 2.8 programme. Neutrophils were gated based on their forward scatter and side scatter profile (Fig 2.1B). Identification of apoptotic cells were performed as shown in Fig 2.2. Percentage of cell survival was quantified by comparing the number of $DiOC_6$ high cells after 20 of culture to that at the start of culture.

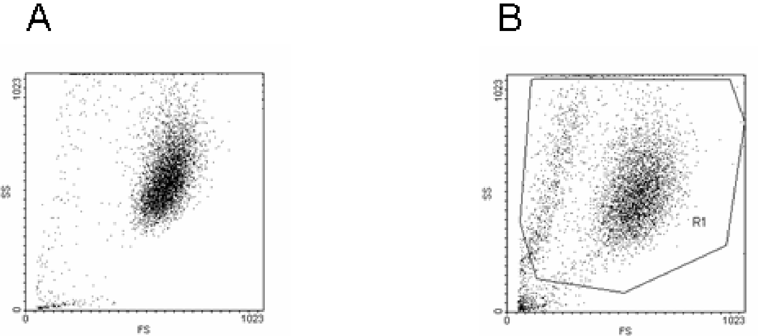


Fig. 2.1 Gating of neutrophils based on their forward scatter vs. side scatter profile A. Dot plot of freshly isolated neutrophils. B. Dot plot of neutrophils after 20h in culture.

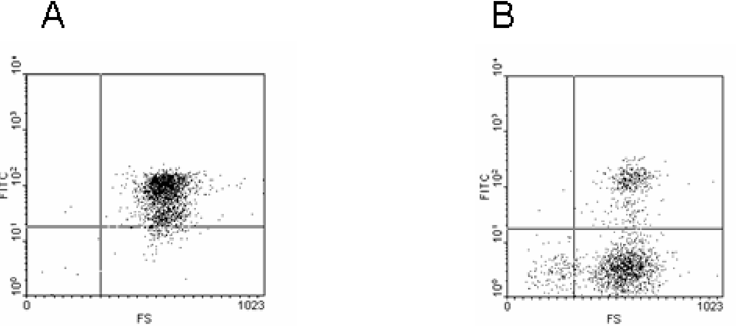


Fig. 2.2 Identification of apoptotic cells using DiOC6 staining. A. Freshly isolated neutrophils. B. Neutrophils after 20 h of culture. Gate set as shown in Fig.2.1B

Neutrophil apoptosis was also assessed by cell morphology. Apoptotic neutrophils can be distinguished by morphological features such as nuclear condensation, loss of connection between lobules of the nucleus and reduced cytoplasm (Savill et al., 1989).

Cytospin slides were differentially stained with Diff Quick and the percentage of apoptotic cells was quantified by blind counting of 200 cells per field under light microscopy (×1000). Three fields of total 600 cells were counted per slide. Results from both methods gave comparable levels of apoptosis.

2.9.2 Fibroblasts apoptosis assay

Synovial fibroblasts transfected with galectin-9 or control siRNA were treated with 5μM staurosporine (Sigma) to induce apoptotic cell death. After 6h cells were detached with accutase (PAA Laboratoories), combined with those floating in the medium and washed with cold PBS. Supernatants were removed and 100μl of Labelling solution (consisting of incubation buffer: 10 mM HEPES, pH 7.4; 140 mM NaCl; 2.5 mM $CaCl_2$ with 2μl Annexin-V-FITC and 2μl propidium iodide) was added to each tube. Cells were incubated for 15 min. at 4^0 C protected from light, then washed once with incubation buffer and analysed using FACS Calibur and CellQuest software. Annexin-V binds to phosphatydylserine which is exposed on cell surface early during apoptotic process. Propidium iodide stains dead cells (late apoptotic and necrotic). Annexin-V-high cells were considered as apoptotic (both early and late).

2.9.3 Fibroblasts proliferation assay

Proliferation of fibroblasts was assessed using an XTT assay (Cell proliferation kit, Biological Industries). This method is based on the ability of live cells to reduce tetrazolium salt XTT into coloured formazan compound. The intensity of the formed dye is proportional to the number of metabolic active cells and can be read using a spectrophotometer. Fibroblasts were cultured on 96-well plates at the density of $8x10^4$ in 100μl of medium. For positive control they were treated with 10ng/ml TNFα and 10ng/ml IL-1β. Cell proliferation was assessed at 24, 48 and 72h after addition of cytokines. XTT substrate was prepared by mixing 5ml of XTT Reagent and 0.1ml Activation Reagent and 50μl of the solution was added into duplicate wells. Plates were incubated at 37^0 C for 3h and read at 450nm with a reference wavelength of 620nm.

2.10 Statistical analysis

Non-parametric distribution was assumed for all assays. For comparison of two groups (eg. expression in RA vs OA) Mann-Whitney test was used. Comparisons across multiple groups (eg. neutrophil survival, fibroblast stimulations, galectin expression in matched fibroblasts) were performed with Kruskal-Wallis one-way analysis of variance and Dunn's post test. The differences between paired samples (eg. depletion and blocking experiments, inhibitors, fibroblasts apoptosis) were assessed with Wilcoxon signed rank test with 2-tailed P values.

RESULTS AND DISCUSSION

3.0 Fibroblast-induced neutrophil survival

3.1 Background

Rheumatoid synovial fibroblasts are key players in the development of the disease. In addition to their aberrant proliferation and invasive properties they also contribute to the pathology indirectly by attracting and supporting the inflammatory infiltrate in the synovial microenvironment. Synovial fibroblasts are a source of a variety of immune mediators with potent activation and anti-apoptotic properties. One of the cell types that may be influenced by these cells are neutrophils. These polymorphonuclear leukocytes persist in the synovial fluid in rheumatoid arthritis (RA) in large quantities, in part because their normal entry into apoptosis is delayed (Edwards and Hallett, 1997). It was hypothesized that factors produced by RA synovial fibroblasts and released into the synovial fluid may be responsible for this effect.

Previous studies from this laboratory have shown that fibroblasts from the rheumatoid joint produce soluble factors able to prolong neutrophil survival in vitro (Filer, 2006). In this chapter the ability of cytokines present within the RA joint to influence fibroblast effects upon neutrophils was determined. Specifically, the effect of pretreatment of fibroblasts with the inflammatory cytokines IL-17 and TNFα was assessed. TNFα is at the top of proinflammatory cascade and is present at high levels in the rheumatoid joint (Brennan et al., 1998). IL-17, a T cell derived cytokine, is also an important factor promoting inflammation in the joint (Chabaud et al., 1999; Lubberts et al., 2005). It has recently been described as part of a transient cytokine profile present in synovial fluid at early stages of RA (Raza et al., 2005a).

Furthermore, both cytokines are potent activators of synovial fibroblasts and can act in a synergistic manner (McInnes and Schett, 2007; Ruddy et al., 2004).

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a well-known neutrophil activation and survival agent (Lee et al., 1993; Kobayashi et al., 2005). It was found previously that GM-CSF partially accounted for the increased survival of neutrophils in medium conditioned by cytokine-activated fibroblasts. However, depletion of this molecule from the conditioned medium did not abolish the neutrophil survival activity completely, reducing it only by around 50% (Filer, 2006). The present series of studies was undertaken to identify the factors secreted by activated fibroblasts that contribute to prolonged neutrophil survival.

3.2 Identification of neutrophil survival factors produced by activated fibroblasts

It was first confirmed that fibroblast-conditioned medium promoted in vitro survival of neutrophils. Synovial fibroblasts were pretreated with the respective cytokines for 24h, then medium containing the stimulants was washed off and cells cultured in medium alone for the next 24h. The latter was collected and will be referred to as “fibroblast-conditioned medium”. Neutrophils isolated from the blood of healthy donors were cultured in the fibroblast conditioned medium or medium with 100pg/ml recombinant GM-SCF as a positive control. Apoptosis of neutrophils was measured after 20h of culture by flow cytometry of $DiOC_6$–stained cells, as a measure of mitochondrial membrane integrity. Cells were also stained with a commercial Giemsa stain (Diff Quick) and examined for morphological changes indicative of apoptosis. Both methods yielded comparable results.

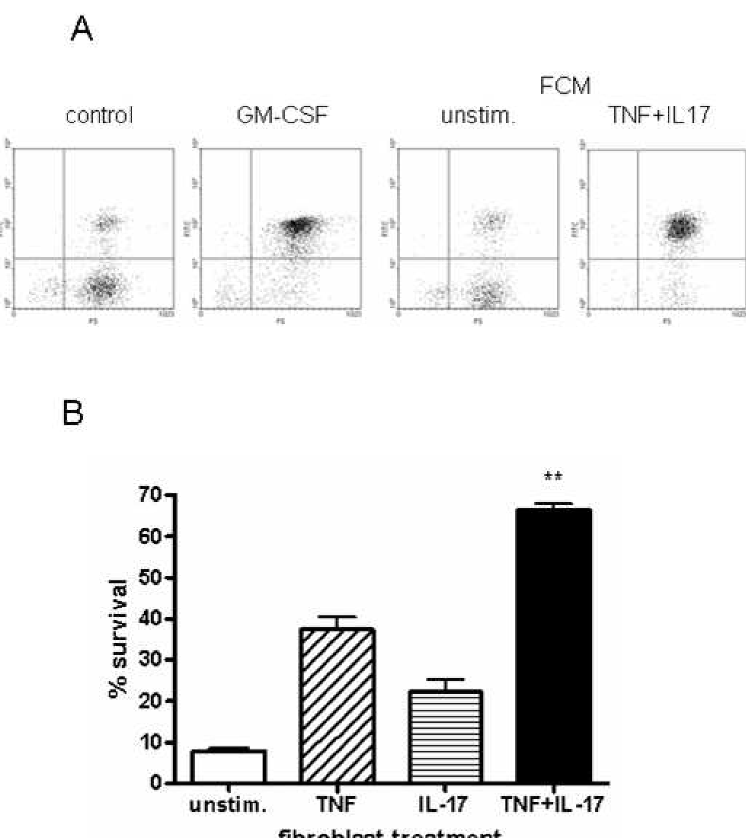


Fig. 3.1. Effect of conditioned media from cytokine-activated fibroblasts on neutrophil survival in culture.

RA synovial fibroblasts were pretreated with 10ng/ml TNFα and/or 10ng/ml IL-17 for 24h. Conditioned media were collected after another 24h and used to culture freshly isolated neutrophils from peripheral blood of healthy donors. Neutrophil survival was assessed after 20h of culture. **A.** Flow cytometry dot plots from a representative experiment. FITC vs forward scatter plots show cells with high (viable) and low (apoptotic) membrane potential after DIOC6 staining. **B.** Summarized data from 4 independent experiments run in duplicate. Survival is expressed as % $DiOC_6$-positive cells as compared to the number at the start of the culture. Bars represent mean +SEM. ** p<0.01 compared to survival in media from unstimulated fibroblasts.

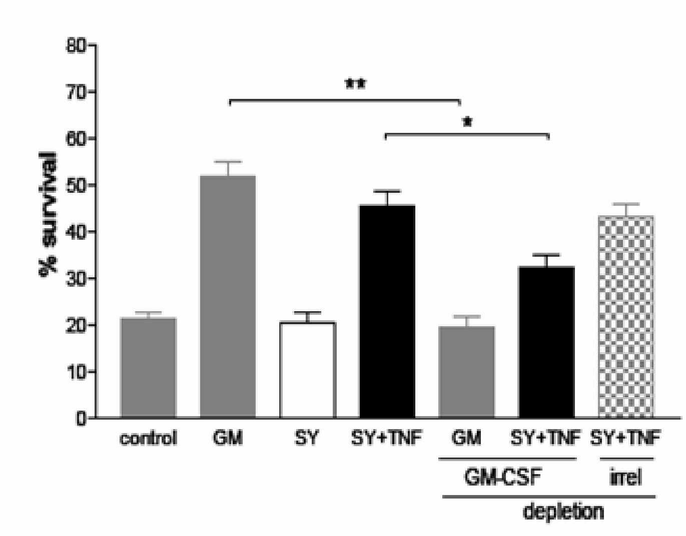


Fig. 3.2 Effect of GM-CSF depletion on neutrophil survival.

Neutrophils were cultured with media alone (control), medium with 100pg/ml GM-SCF (GM), fibroblast-conditioned media from unstimulated cells (SY), fibroblast conditioned media from cells treated with TNF and IL-17 (SY+TNF). Additionally, conditioned media were depleted with anti-GM-CSF antibody or control IgG (irrel). Apoptosis was measured by DiOC6-staining. Bars represent means +SEM of triplicate assays from 4 independent experiments. * $p<0.05$, ** $p<0.01$ comparison between indicated bars. *Figure taken from (Filer, 2006).*

Conditioned media from cytokine-activated fibroblasts extended neutrophil survival significantly, reducing apoptosis after 20h by approximately 60%. The survival effect was comparable to that seen with GM-CSF, but media from unstimulated fibroblasts had no significant effect on neutrophil survival (Fig 3.1A). The results also showed that combined pre-treatment of fibroblasts with TNFα and IL-17 produced a medium that induced much greater neutrophil survival than medium from cells treated with a single treatment alone (Fig. 3.1B.)

As potential factors contributing to prolonged neutrophil survival several inflammatory mediators were tested including: GM-CSF, IFNβ, IL-6, TNFα and galectin-3. Figure 3.2 shows that depletion of GM-CSF from the conditioned medium reduced the survival effect by approximately 50% (Filer, 2006) and suggesting that other survival factors were also present in the medium.

It was reported previously that IFNβ produced by stromal cells mediates T cell rescue from apoptosis (Pilling et al., 1999). It can also delay apoptosis of neutrophils in vitro (Scheel-Toellner et al., 2002; Wang et al., 2003). Because it was not possible to directly measure IFNβ levels in fibroblast-conditioned media, another approach was taken to test whether it contributed to the prolonged survival of neutrophils observed in the fibroblast conditioned medium. After isolation neutrophils were pre-treated with a blocking antibody against type I IFN receptor (CD118) for 1h and then cultured for 20h in either medium alone, medium with recombinant IFNβ (800U/ml) or fibroblast-conditioned medium (from non-stimulated or IL-17+TNFα-stimulated cells). As a control untreated neutrophils were cultured in the same conditions. Because the presence of receptors for Fc fragments of immunoglobulins on neutrophils could cause non-specific activation and therefore affect survival of

neutrophils an additional control was added, namely cells that were pre-treated with an irrelevant IgG of the same isotype. As shown in Fig. 3.3, pre-treatment of neutrophils with anti-CD118 receptor antibody effectively blocked the survival effect of recombinant IFNβ but did not affect the survival of neutrophils in fibroblast-conditioned medium. There was no effect on cell survival from the exposure to an isotype matched irrelevant antibody. This result suggests that IFNβ is unlikely to be present in IL-17-TNFα fibroblast-conditioned media at a level that could account for increased neutrophil survival.

Another cytokine that is produced in large quantities by synovial fibroblasts is IL-6. Its level in media collected from fibroblasts after their activation by IL-17 and TNFα was greatly increased as measured by ELISA (Fig.3.4). The role of IL-6 as a neutrophil survival factor remains controversial. Although some reports show its possible anti-apoptotic action towards neutrophils (Daffern et al., 1999), in these experiments no such effect was observed. Recombinant IL-6 was used across the range of concentrations found in fibroblast-conditioned media (Fig.3.4) but failed to rescue neutrophils from apoptosis when added to culture medium (Fig.3.5). Since the recombinant protein might behave in a different way to the natural cytokine present in the supernatant together with other factors, a receptor blocking experiment was also performed. Neutrophils were pre-treated with an anti-IL6R antibody or irrelevant IgG and then cultured with or without IL-6 or in conditioned media. No reduction in survival rates in the TNF-IL17 conditioned medium was noted ruling out the possibility of IL-6 contributing to the survival effect (Fig.3.6).

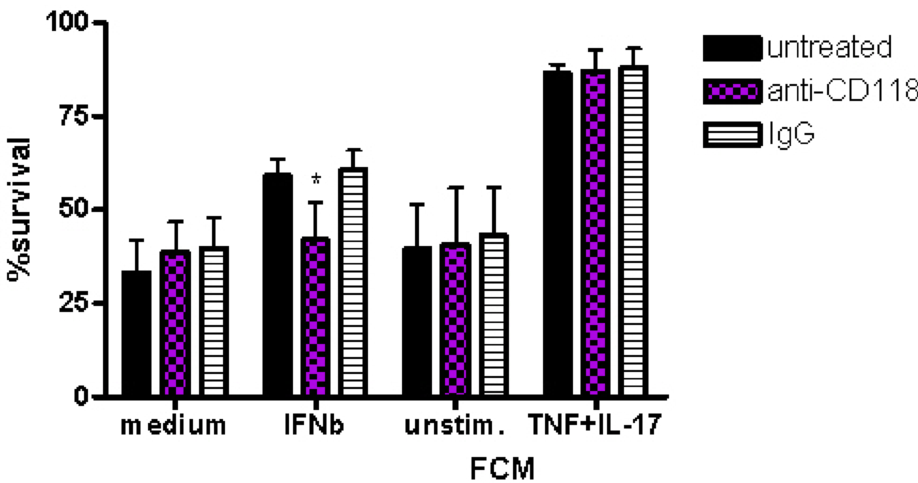


Fig. 3.3. Blocking of IFN receptors on neutrophils - effect on survival induced by recombinant IFNβ and fibroblast-conditioned media.

Neutrophils were pretreated with anti-CD118 blocking antibody or irrelevant IgG for 1h and then cultured with medium alone, 800U/ml recombinant IFNβ or fibroblast-conditioned media (FCM) from unstimulated or TNF+IL-17-treated cells. Apoptosis was assessed after 20h of culture. Bars represent mean +SEM from 3 independent experiments run in duplicate. * $p<0.05$ compared to survival in medium alone.

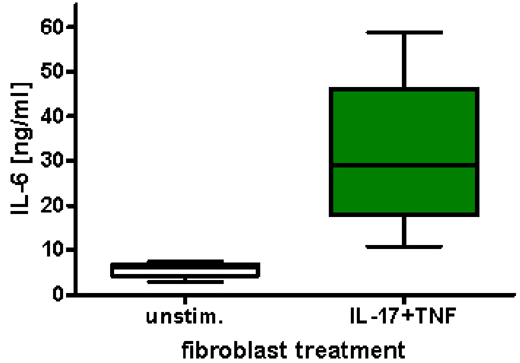


Fig. 3.4 Interleukin-6 levels in fibroblast-conditioned media.

Synovial fibroblasts were treated with 10ng/ml TNFα and IL-17 for 24h, then medium was exchanged and collected after another 24h. The level of IL-6 in conditioned media was measured by ELISA. Boxes show mean and 5%-95% percentile, whiskers show range.

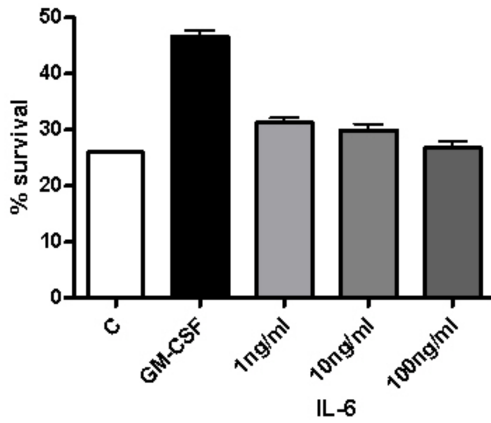


Fig. 3.5 Effect of recombinant IL-6 on neutrophil survival.

Neutrophils were cultured in vitro with 100pg/ml GM-CSF, 1, 10 or 100ng/ml IL-6. Survival was assessed after 20h with DiOC6 staining. Bars represent mean +SEM from 3 independent experiments.

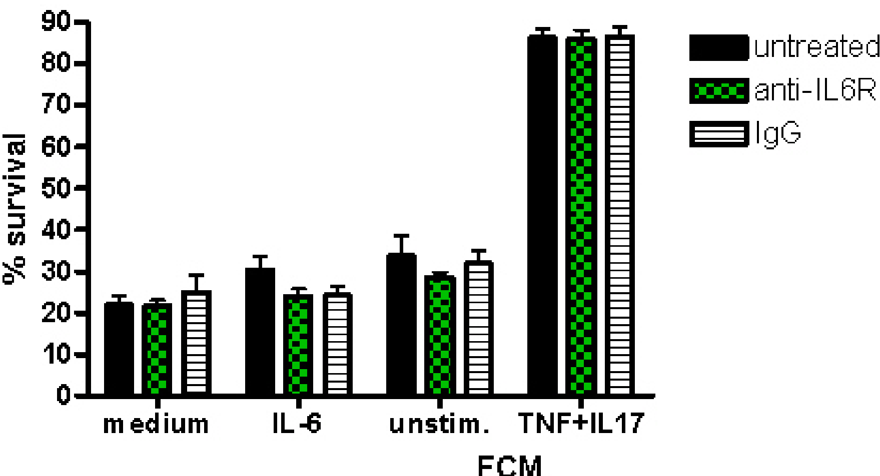


Fig. 3.6. Blocking of IL-6 receptors on neutrophils - effect on survival induced by recombinant IL-6 and fibroblast-conditioned media.

Neutrophils were pretreated with anti-IL6R blocking antibody or irrelevant IgG for 1h and then cultured with medium alone, 10ng/ml recombinant IL-6 or fibroblast-conditioned media (FCM) from unstimulated or TNF+IL-17-treated cells. Apoptosis was assessed after 20h of culture. Bars represent mean +SEM from 3 independent experiments run in duplicate.

Another possible factor that was tested was TNFα. This cytokine has been described to have a dual role in the regulation of neutrophil survival, either protecting them or inducing apoptosis (van den Berg et al., 2001; Walmsley et al., 2004). To assess the role of TNFα for the survival of neutrophils in our experimental setting, cells were cultured with different concentrations of the recombinant protein. In contrast to the study by van den Berg et al. where anti-apoptotic effect was observed at concentrations lower than 1 ng/ml, here it was protective only at high concentrations (100ng/ml) (Fig. 3.7A). To examine if the presence of TNFα in fibroblast-conditioned media might contribute to its survival-promoting effect, depletion of this cytokine was performed. Fibroblast-conditioned media (FCM) were incubated with agarose beads bound to anti-TNFα antibody. The beads with captured cytokine were then removed by centrifugation at high speed. Additionally depletion with an irrelevant control antibody was performed. To ensure the efficiency of the depletion the same procedure was applied to medium with exogenously added 100ng/ml recombinant TNFα. As can be seen in Fig. 3.7B there was a significant reduction in TNF-induced cell survival if the medium was depleted of TNFα. Depletion of TNFα had no effect on the survival effect induced by fibroblast conditioned medium (Fig. 3.7B). Finally, to assess whether GM-CSF may synergize with any of the tested cytokines (IFNβ, IL-6 or TNFα) in supporting neutrophil survival, an assay combining all the treatments was performed. In this case neutrophils were first pre-treated with both anti-CD118 and anti-IL6R antibodies and then cultured in FCM depleted of GM-CSF and TNFα. Although all depletions and blocking experiments were efficient as observed in single experiments, there was no additive effect of the combined treatment (Fig.3.7B), indicating that none of these factors contributes to survival promoted by GM-CSF.

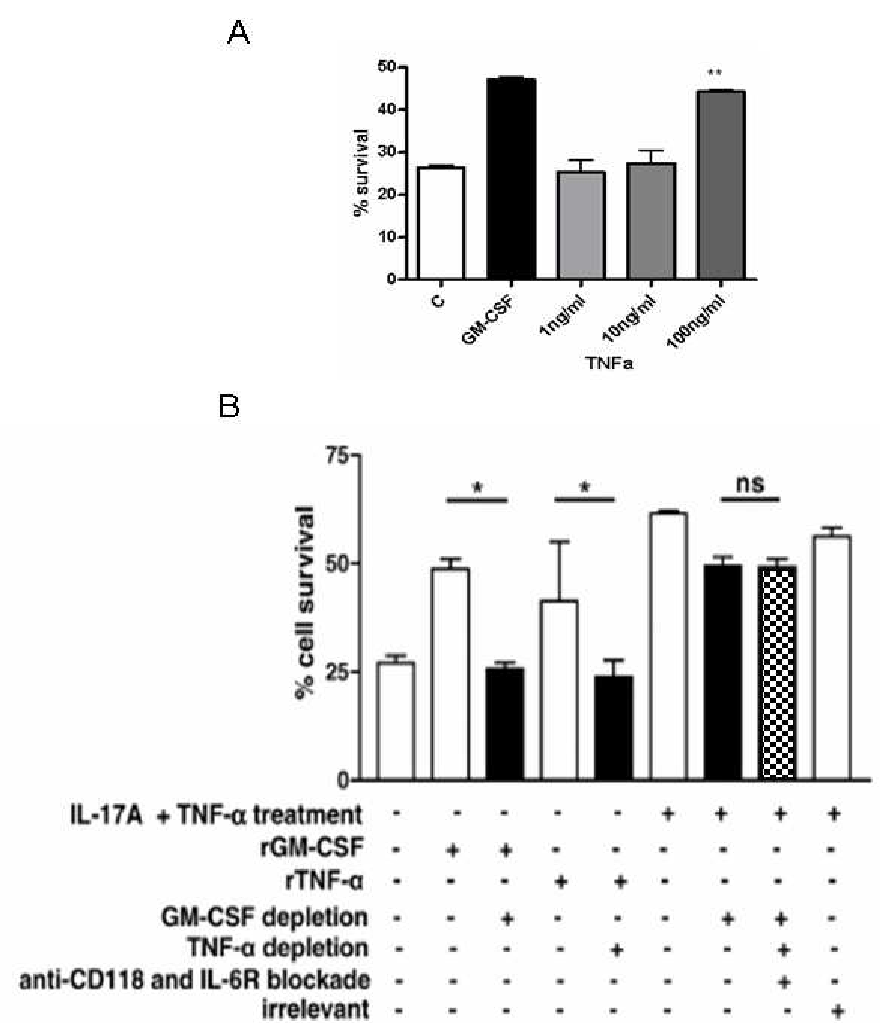


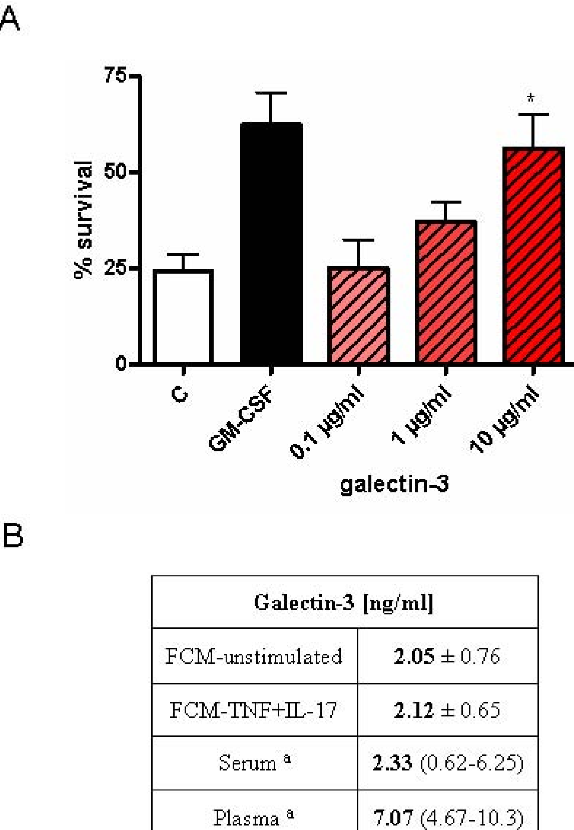
Fig. 3.7 Effect of recombinant TNFα (A) and depletion of TNFα and/or GM-CSF from fibroblast-conditioned media combined with receptor blocking on neutrophils (B) for their survival.

A.Neutrophils were cultured in vitro with 100pg/ml GM-CSF, 1, 10 or 100ng/ml TNFα. **B.** Neutrophils were cultured in medium containing 100pg/ml GM-CSF or 100ng/ml TNFα. Depletion was performed with anti-GM-CSF and anti-TNF antibodies, respectively. Fibroblast-conditioned media were depleted from both cytokines and used on neutrophils that were pre-treated with anti-CD118 and anti-IL6R antibodies or with irrelevant IgG. Survival was assessed after 20h with DiOC6 staining. Bars represent mean +SEM from 3 experiments run in duplicate. * $p<0.05$, **$p<0.01$ compared to control (part A) or between indicated bars (part B).

3.3 Role of fibroblast-produced galectin-3 in neutrophil survival

Galectin-3 is an immunomodulatory lectin identified in many inflammatory conditions. It is known to modulate the survival and apoptosis of different cell types (Hsu et al., 2006). Secretion of galectin-3 from rheumatoid synovial fibroblasts and its elevated levels in synovial fluid are already described in RA patients (Ohshima et al., 2003). Therefore it was hypothesized that galectin-3 might act as a survival factor for neutrophils and be present in the FCM.

In order to test this possibility neutrophils were cultured with different concentrations of the recombinant protein and then their apoptosis was measured as previously by $DiOC_6$ staining. Galectin-3 prolonged neutrophil survival in culture in a dose-dependent manner (Fig.3.8A). However, when the levels of galectin-3 in fibroblast-conditioned media were measured, there was no difference between unstimulated and TNFα+IL-17-treated cell media (Fig.3.8B). Moreover, the levels observed in the supernatant were much lower that those required to effectively delay neutrophil apoptosis in vitro (Fig. 3.8A), although they were in the range found commonly in serum. It was concluded that galectin-3 is unlikely to mediate the enhanced neutrophil life span observed in media from cytokine activated fibroblasts.



Galectin-3 [ng/ml]	
FCM-unstimulated	**2.05** ± 0.76
FCM-TNF+IL-17	**2.12** ± 0.65
Serum [a]	**2.33** (0.62-6.25)
Plasma [a]	**7.07** (4.67-10.3)

Fig. 3.8 Effect of recombinant galectin-3 on neutrophil survival and levels of galectin-3 in fibroblast-conditioned media.

A. Neutrophils were cultured with 100pg/ml GM-CSF, 0.1, 1, 10 µg/ml recombinant galectin-3. Apoptosis was assessed after 20h of culture. Bars represent mean +SEM from 5 independent experiments run in duplicate. * $p<0.05$ compared to survival in medium alone (C - control). **B**. Levels of galectin-3 in FCM measured by ELISA. Results are mean ± SD from 3 different experiments. a-Median values and ranges for human serum and plasma taken from ELISA kit datasheet.

3.4 DISCUSSION

Our studies revealed that cytokine activation of RA synovial fibroblasts leads to the production of soluble factors able to delay neutrophil apoptosis. TNFα and IL-17 act in a synergistic manner on fibroblasts to induce those factors. GM-CSF is partially responsible for neutrophil rescuing effect, the rest of it could not be accounted for by IFNβ, IL-6, TNFα or galectin-3.

Accumulation of neutrophils in the joint of rheumatoid arthritis patients is a phenomenon that is not fully understood. One reason for this observation may be their enhanced recruitment from the circulation which fails to be down-regulated in chronic inflammation (Wong and Lord, 2004). Another is their prolonged survival in the diseased joint microenvironment. The persistence of this inflammatory infiltrate is important in RA pathology as it may lead to increased tissue damage by activated neutrophils and a failure of inflammation to resolve (Edwards and Hallett, 1997; Cross et al., 2006). The literature suggests that extended neutrophil life span is likely to result in part from interactions with synovial stromal cells. It was demonstrated that ligation of α_9 β_1 integrin on the surface of neutrophils by vascular endothelial adhesion molecule (VCAM-1) provides survival signal for these cells (Ross et al., 2006). Also other reports show that modulation of apoptotic death in neutrophils depends on their adhesion to subendothelial matrix after extravasation (Watson et al., 1997; Ginis and Faller, 1997).

However, most neutrophils in the joint migrate through the tissue and are located in the synovial fluid without direct cell-cell contact with other cell types. Therefore soluble factors are likely to play a major role in maintaining them within the joint for extended periods of time. Typically the main sources of inflammatory cytokines in

the environment of a rheumatoid joint are fibroblasts and macrophages. Some fibroblasts even in the absence of any stimulation were shown to produce neutrophil survival factors (Southey et al., 1994). Rheumatoid synovial fibroblasts are likely to be constantly activated by cytokines present in the synovium such as TNFα or IL-17 (Miossec, 2003) and the role of such processes was considered here.

A number of papers showed synergistic effects of these two cytokines in stimulating RA synovial fibroblasts. Such activation led to increased production of IL-6, IL-8 and IL-1 (Katz et al., 2001). A specific synergism between TNFα and IL-17 in fibroblasts was shown here and is in agreement with the data of Goldberg et al. (Goldberg et al., 2009). These authors proposed that IL-17 acted on fibroblasts as a priming agent making them hyperresponsive to TNFα. A few studies showed that fibroblasts can increase the production of potential neutrophil survival factors (GM-CSF, G-CSF, IL-6) upon stimulation with IL-17 (Fossiez et al., 1996; Laan et al., 2003). Elaboration of neutrophil survival factors as a result of TNFα and IL-17 stimulation was also shown in other cell types, such as epithelial, and contributed to persistence of neutrophil infiltrate in the affected organs (Daffern et al., 1999; Laan et al., 2003).

The finding initially made by our group (Filer, 2006) and confirmed in the present study that synovial fibroblasts produce neutrophil survival factors in response to TNFα and IL-17 therefore agrees with the previous observations. GM-CSF was found to account for part of this effect. A role for this cytokine in accumulation of neutrophils was shown in the lung where it was induced from bronchial epithelial cells by TNFα and IL17 (Laan et al., 2003; Numasaki et al., 2004). Here we tested other putative factors that could be responsible for delaying neutrophil apoptosis

such as IFNβ, IL-6 and TNFα. Although the anti-apoptotic function of the latter two is a matter of debate in the literature with some studies showing the opposite actions (Salamone et al., 2001), we found that TNFα, but not IL-6, could delay neutrophil apoptosis in our culture system. Recombinant IL-6 had no effect on the rate of neutrophil cell death.

The overall actions of these two cytokines are probably more complex than being simply pro- or anti-apoptotic and may largely depend on other signals present in the environment, on activation status of the cell or, in vitro, on a particular experimental setup. However, they do not seem to play a role in neutrophil rescue from apoptosis exerted by fibroblast-conditioned medium as shown by depletion and blocking experiments. Other candidate survival factors that were found at elevated levels in FCM, such as G-CSF, CCL2 (MCP-1) or CXCL8 (IL-8), were tested previously but did not add to the effect of GM-CSF (Filer, 2006; Parsonage et al., 2008). That study also excluded a role for PGE_2, a lipid mediator that may delay neutrophil apoptosis and whose production by fibroblasts is augmented after TNFα and IL-17 treatment (Fossiez et al., 1996)

Despite the reports by Ottonello (Ottonello et al., 2002) about anti-apoptotic effects of adenosine, a nucleoside present in the synovial fluid, in our hands this substance did not show any effect towards neutrophils (A. Filer, personal communication). It is however important to stress that different agents may exert their role only in certain conditions, such as low oxygen tension. The synovial microenvironment is hypoxic (Gaber et al., 2005) and, as was demonstrated by Cross et al., that determines the action of various factors from the synovial fluid on neutrophil apoptosis (Cross et al., 2006). Indeed, studies performed under hypoxic conditions identified novel

fibroblast-derived neutrophil survival factors such as CCL4 (MIP-1β) (Walmsley et al., 2005).

Although this situation does not apply to experiments with fibroblast-conditioned media as they were all performed in standard cell culture conditions (i.e. atmospheric oxygen levels), it underlines that caution should be taken when extrapolating results obtained in vitro to the situation that occurs in vivo. Another factor that may account for possible differences with the actual in vivo situation is the fact that all our studies used peripheral blood and not synovial fluid neutrophils. As already mentioned the transmigration through the endothelium may itself alter the survival properties of neutrophils (Hu et al., 2005) as compared to naïve cells.

Another immunomodulatory protein that was of interest in our study was galectin-3. High expression of galectin-3 in the rheumatoid synovium and its release in soluble form by RA synovial fibroblasts was reported in a study by Ohshima et al. Moreover, the production of galectin-3 in RA synovium was much higher compared to OA or healthy controls (Ohshima et al., 2003). As neutrophil apoptosis is delayed in the RA synovium but not the OA synovium (Raza et al., 2006) this could indicate a role for this lectin in extended neutrophil life span in RA synovial fluid. The effects of galectin-3 on neutrophils that have been described so far include promotion of neutrophil adhesion to laminin (Kuwabara and Liu, 1996), facilitation of extravasation (Leffler et al., 2004) and activation of NADPH-oxidase leading to respiratory burst (Karlsson et al., 1998). Galectin-3 may also act on naïve and primed neutrophils to induce events such as L-selectin shedding and production of IL-8 (Nieminen et al., 2005). The role of galectin-3 in survival/apoptosis of neutrophils has not been studied to date.

Although several reports show pro-apoptotic action of exogenous galectin-3 on some cells (leukaemia T cells lines and peripheral blood mononuclear cells), there is also evidence of galectin-3 as a mitogen (Inohara et al., 1998), suggesting that its extracellular action does not necessarily trigger cell death.

The present study showed that galectin-3 at high concentrations could rescue neutrophils from apoptosis in vitro. However, there are some issues which have to be considered before drawing conclusions about the relevance of this finding for the in vivo situation. First is whether the observed survival effect is directly induced by galectin. Two molecules, CD66a and CD66b, were previously identified as surface receptors for galectin-3 on neutrophils (Feuk-Lagerstedt et al., 1999). It was demonstrated that interaction of galectin-3 with CD66b molecule leads to a release of pre-formed IL-8 from neutrophil granules (Schroder et al., 2006). Intracellular stores of this chemokine can be mobilized during exudation from the vessels which would help to establish a chemotactic gradient for other neutrophils (Pellme et al., 2006) but in naïve cells this is unlikely to happen. It might be that release of this chemokine induced by cross-linking of the respective receptors by galectin-3 accounts for the observed rescue from apoptosis. A pro-survival effect of IL-8 on neutrophils has been demonstrated elsewhere (Dunican et al., 2000; Kettritz et al., 1998).

The possibility that enhanced survival was mediated by a contaminating agent in the recombinant protein such as lipopolysaccharide (LPS) was also excluded. Because galectin-3 has high affinity for bacterial LPS it is of particular importance that all galectin preparations are endotoxin-free (Sarter et al., 2009). Since LPS is a potent activator for neutrophils even small amounts could give survival effect. We excluded

the possibility that the galectin-3 used was LPS-contaminated (endotoxin levels <0.1 EU/ml as measured by Limulus amebocyte assay).

Another issue is a possible neutralisation of galectin-3 that may occur in vivo. Study by Nieminen et al. showed that primed neutrophils are capable of cleaving galectin-3 by elastase which causes inactivation of this protein (Nieminen et al., 2005). In the present study all experiments were performed with freshly isolated neutrophils which by definition are naïve. Standard isolation procedures should not activate the cells and any preparations showing signs of neutrophil activation (as assessed by altered morphology, spreading and clumping) were excluded. Galectin-3 treatment was able to extend the life span of naïve neutrophils, but whether similar effect would be observed in vivo with cells that have been primed is not clear.

In our study the survival effect was observed with relatively high concentrations of galectin. It is possible that such concentrations are required for effective cross-linking of surface receptors. Extracellular fluids at inflammatory sites may contain high levels of galectin-3 (Farnworth et al., 2008). The range noted in serum and synovial fluid of RA patients was 30-100ng/ml and 150-300ng/ml, respectively (Ohshima et al., 2003). However in media collected from cytokine-activated fibroblasts the level was much lower (2 ng/ml) and not different from that in non-treated cells ruling out the possibility that galectin would contribute to neutrophil survival in this experimental setting.

In summary, previous study by A. Filer (2006) as well as the present one eliminated a number of candidate neutrophil survival factors. Which other soluble molecules, apart from GM-CSF, are secreted by fibroblasts activated with TNFα and IL-17 that can delay neutrophil apoptosis remains to be established.

4.0 Expression of galectins and their regulation in fibroblasts

4.1 Background

Galectins are important modulators of cellular processes such as survival, apoptosis, adhesion or activation. As they affect many immune cell functions, galectins may be involved in immunoregulation elicited by stromal fibroblasts.

One of the aims of this project was to establish a profile of galectin expression in fibroblasts from different anatomical sites with special regard to fibroblasts from patients with rheumatoid arthritis. This was necessary to determine whether there are differences in production of these lectins among fibroblasts which might contribute to site-specific differences in supporting immune cell survival.

Numerous papers show galectin involvement in chronic inflammation (reviewed by (Rabinovich and Toscano, 2009). Galectin-1 administration reduced the severity of inflammation in several model of experimental inflammation (Rabinovich et al., 1999; Offner et al., 1990, Santucci et al., 2000). Galectin-3 on the other hand appears to promote inflammation by various mechanisms described previously. Its high levels in serum was reported in inflammatory bowel disease, Behcet's disease, rheumatoid arthrits, as well as various cancers and correlated with disease severity (Frol'ova et al., 2009; Iurisci et al., 2000; Lee et al., 2007; Ohshima et al., 2003; Vereecken et al., 2006). Intestinal inflammation is another condition where galectins are important factors. Galectin-1 and -2 were shown to have a suppressive role in this disease by promoting T cell apoptosis, while galectin-4 exacerbated the inflammatory state via stimulation of CD4+ cells (Hokama et al., 2008). Galectin-9 is

also able to modulate autoimmune arthritis by selective reduction of Th17 responses and induction of regulatory T cells (Seki et al., 2008).

Fibroblasts are known to produce many immunoregulatory mediators and may be an important source of galectins. It was previously shown that fibroblasts depending on their site of origin differentially regulated leukocyte survival (Filer et al., 2006a). For example, synovial fibroblasts supported the survival of T cells much better than those from the skin. That study also revealed galectin-3 as one of the classifiers in microarray gene expression analysis of fibroblasts from various sites (Filer, 2006)

4.2 Expression of galectins in fibroblasts

So far fibroblasts were reported to express galectin-3 and, upon activation, galectin-9 (Hirashima et al., 2004; Asakura et al., 2002). Galectin-1 and -8 transcripts were found in extracts from multiple organs including heart, liver, prostate or thymus, but the specific cell types that express them were not identified (Rabinovich et al., 2002). Here seven primary fibroblast lines were screened for the expression of human galectins: 1 through 12. Galectin-13 was not considered here as it is considered only as a placental protein (Than et al., 1999). Two other cell lines: HT-29 (colon adenocarcinoma expressing galectin-2, -3, -4, -7, -8, -9) and HL-60 (acute myeloid leukaemia expressing galectin-1, -3, -8, -9, -10, -12) were used as positive controls. All fibroblast cell lines were grown in standard conditions and used between passage 4-6. RNA extracts were treated with DNase to ensure that no contaminating cellular DNA was present. After conversion to cDNA PCR reactions with specially designed primers (Table. 2.2) were performed. PCR without RT yielded negative results.

Figure 4.1 shows representative images of the PCR amplification results. It was found that mRNA for galectin-1, -3 and -8 was present in all tested fibroblast samples. None of the samples had detectable levels of galectin-2 or -10, although both products were present in the positive controls HT-29 or HL-60, respectively (Fig. 4.1). Galectin-4 was not expressed by fibroblasts from spleen, lung, tonsil and thyroid. However, it was present in synovial, bone marrow and skin fibroblasts from RA patients, although the expression pattern varied between individuals. Galectin-7 product could be seen in some samples only with a high number of rounds of PCR amplification (38 cycles), therefore it was uncertain if this was an artefact or not. With the exception of tonsilar fibroblasts, galectin-9 mRNA was detected in all other fibroblast types. Similar to galectin-4, there was significant variation among different individuals. Galectin-12 mRNA was detected in some synovial samples but not bone marrow or skin (Figure 4.1).

As the traditional end-point PCR reaction is not accurate enough to measure quantitative differences in gene transcription products, all galectins were also analysed by the real-time PCR method in 4-6 matched fibroblast cell lines. Galectin-7 expression was also checked by this method since the results obtained previously were rather inconclusive. Real-time PCR revealed high levels of galectin-1 and -3 mRNAs in synovial, bone marrow and skin fibroblasts (Fig 4.2A). Expression of galectin-4 in all samples from five matched RA fibroblast lines (synovium, bone marrow, skin) was confirmed, but no significant site-specific differences in its expression were detected. Similar results were seen with galectin-8. Galectin-9 was present at all three sites. Interestingly, its level in synovial fibroblasts was significantly higher compared to bone marrow or skin fibroblasts (Fig.4.2B). Real-

time PCR revealed the presence of galectin-12 mRNA in synovial fibroblasts from four out of six cell lines tested, but the levels of expression were quite variable. Skin fibroblasts did not show any expression of this galectin (Fig 4.2A). Due to lack of information on clinical parameters of patients from whom the biological material came, it was not possible to check whether the presence of galectin-12 in synovial fibroblasts correlated with any specific characteristics of those patients.

It was also confirmed that galectins 2, 7 and 10 were not expressed by synovial, bone marrow or skin fibroblasts. It is worth noting that the real quantities of galectin transcripts were very different for each galectin. For example, the highest level of mRNA was noted for galectin-1 and galectin-3. The amount of galectin-8 was 10 times lower than that of galectin-3 and galectins -4, -9 and -12 were about three logs lower than galectin-3 (Fig. 4.2A).

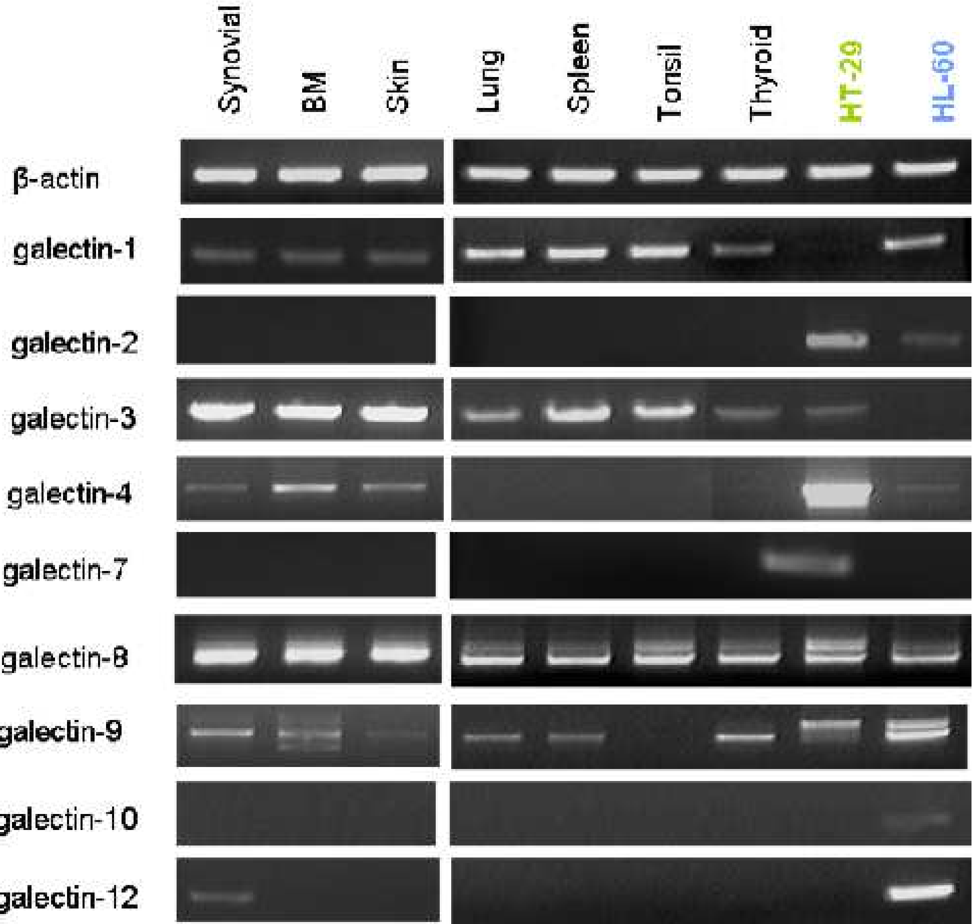
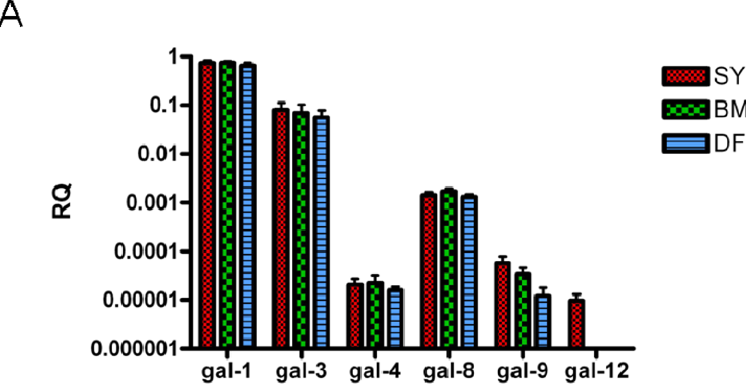


Fig. 4.1 Expression profile of galectins in fibroblasts of different origin.

All fibroblasts were cultured in complete fibroblast media in standard conditions. Synovial, bone marrow and skin fibroblasts were from RA patients. Total RNA was extracted and reverse transcription-PCR reactions performed. Products were separated on agarose gel and visualised with ethidium bromide. Amplification of β-actin was used to ensure cDNA presence in all samples.

A



B

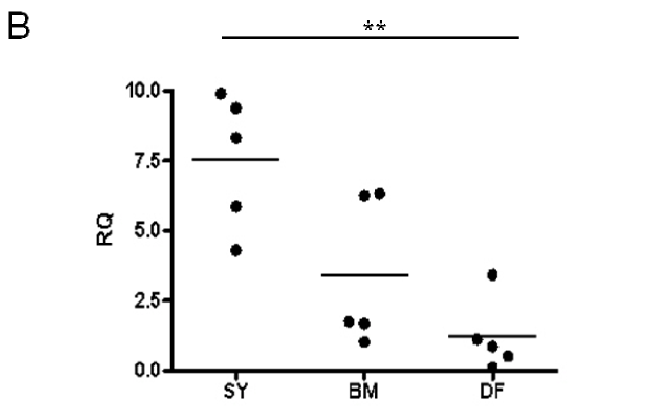


Fig. 4.2 Quantitative differences in galectins mRNA expression in RA fibroblasts.

A. cDNA from matched lines of synovial (SY), bone marrow (BM) and skin (DF) fibroblasts taken from 4-6 different RA patients was amplified using specific TaqMan probes. Bars represent relative quantities of target genes normalized to β-actin and presented on log scale. **B.** Dot plot showing differences in galectin-9 expression in fibroblasts from different sites. Relative expression of galectin-9 mRNA was quantified by real-time PCR in synovial (SY), bone marrow (BM), and skin (DF) fibroblasts from 5 matched lines, ** $p<0.01$

4.3 Expression of galectin-3 and -9 in synovial fibroblasts and tissue

Galectins 3 and 9 were chosen for further study. Expression of galectin-3 in inflamed synovium was reported previously (Ohshima et al., 2003) and galectin-9 was potentially interesting because of its site-specific expression with higher levels in fibroblasts from RA synovium. Since fibroblasts from rheumatoid arthritis synovium differ in many respects from those derived from osteoarthritis patients, they were compared for the expression of galectin-3 and -9.

Synovial fibroblasts from 12 RA and 9 OA patients were grown in culture and then analysed by real-time PCR for RNA expression and by Western blot for protein expression of galectin-3 and -9. There were no significant differences in the mRNA levels of these two galectins between RA and OA fibroblasts (Fig. 4.3 A and C). Both types of fibroblasts in culture had considerable amounts of intracellular galectin-3 and much lower levels of galectin-9 which was consistent with RNA levels. Although OA fibroblasts showed greater variation in protein levels of galectin-9 there was no disease-specific pattern in their expression (Fig.4.3 B and D).

Next the expression of galectins in situ in RA and OA synovial tissue sections was examined. Synovial tissue specimens were fixed in 4% paraformaldehyde, embedded in paraffin and cut into 3 μm sections. Immunohistochemical staining was performed with specific anti-galectin antibodies (Table 2.1). Synovial tissue from rheumatoid arthritis patients showed strong galectin-3 expression throughout the expanded lining and sublining layers (Fig. 4.4 A). Osteoarthritic synovium also stained positively for galectin-3 but due to the lower cellularity of the tissue the staining appeared less intense (Fig. 4.4 E). To identify which cells express galectins double staining procedures were performed.

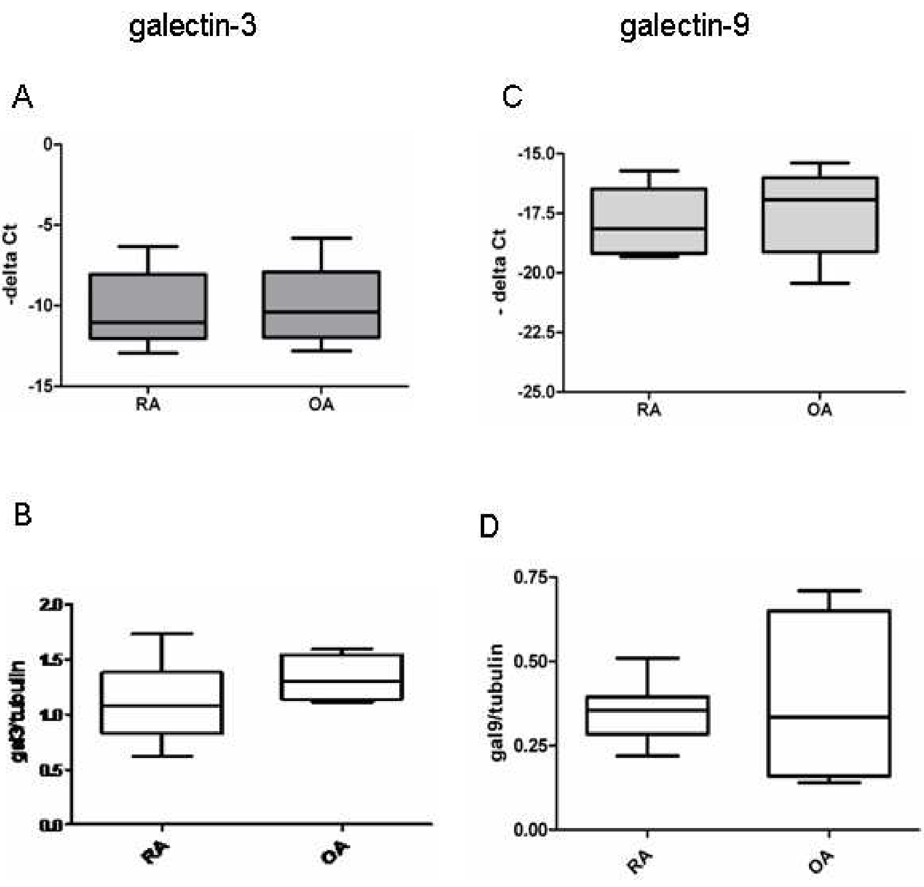


Fig. 4.3 Galectin-3 and -9 expression in fibroblasts from RA and OA patients. Upper panel (grey boxes) shows mRNA level measured by real-time PCR with specific Taqman probes normalized to 18S. Lower panel (white boxes) shows protein level measured by Western blot and expressed as ratio galectin/α-tubulin. **A-B**. Galectin-3 **C-D**. Galectin-9. Boxes represent 5-95 percentile, whiskers show range.

Prolyl-hydroxylase-β was used as a marker for fibroblasts. This enzyme catalyses hydoxylation of proline residues during biosynthesis of collagen and is expressed mostly by fibroblasts. RA tissue displayed stronger staining for fibroblasts than OA confirming marked hyperplasia of these cells (Fig 4.4 B and F), but in both cases galectin-3 co-localized with the fibroblast marker (Fig. 4.4 C and G).

To assess galectin-3 expression in macrophages, double staining with the macrophage surface marker CD68 was performed. The antibody clone used (PG-M1) specifically recognizes CD68 molecule present in macrophages. Double positive cells were seen in RA synovium, mostly in the sublining layer (Fig. 4.5). In OA tissue hardly any macrophages were observed which is in agreement with the general lack of subintimal cellular infiltration in this disease.

Galectin-9 both in RA and OA was expressed in cells of the lining and scattered cells in the sublining, particularly around the microvessels (Fig. 4.6 and 4.8). Galectin-9-expressing cells could be identified as fibroblasts (Fig. 4.6 B and D), macrophages (Fig. 4.7) and endothelial cells (Fig. 4.8 B, C and E) In addition, strong galectin-9 expression in RA synovial tissue was observed at sites of invasion into the cartilage and subchondrial bone (Fig. 4.9) Interestingly, in contrast to galectin-3, galectin-9 often displayed nuclear localization (Fig. 4.6 D, 4.8 C and E).

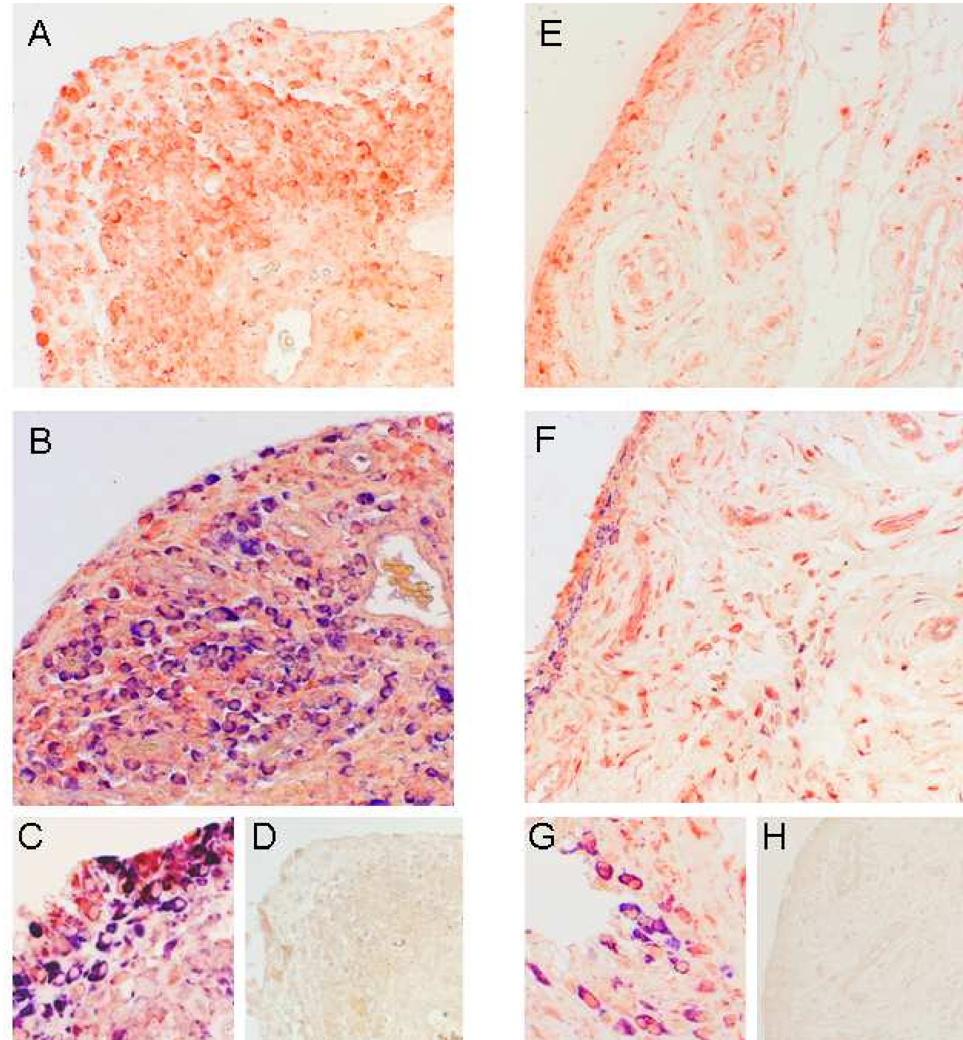


Fig. 4.4 Immunohistochemical detection of galectin-3 in synovial tissues from RA and OA patients – fibroblasts.

Left panel (A-D) shows RA, right panel (E-H) OA synovia. Tissue sections were stained with anti-galectin-3 antibodies (red) (**A**, **E**), with anti-galectin-3 (red) and anti-prolyl hydroxylase (blue) (**B-C**, **F-G)** or with irrelevant IgG of the same isotype as controls (**D, H).** Original magnification x200, C and G x400.

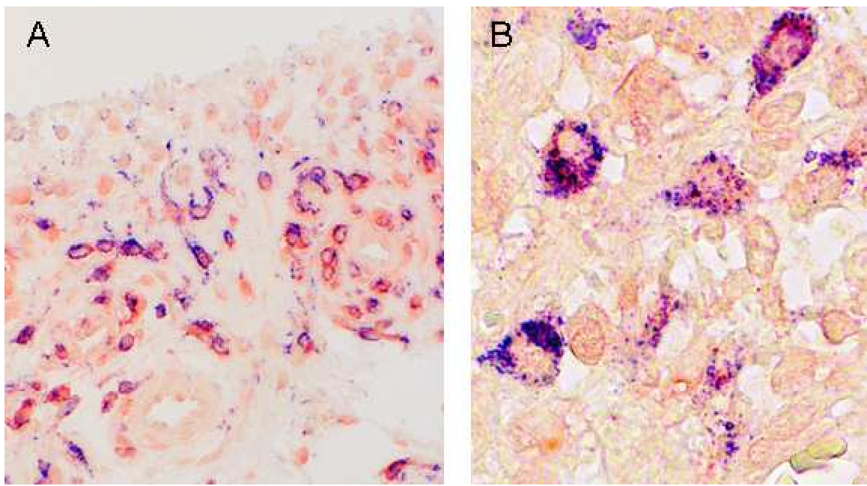


Fig. 4.5 Detection of galectin-3 in RA synovial tissue – macrophages.

Tissue sections were stained with anti-galectin-3 antibodies (red) and anti-CD68 (blue). **A.** magnification 200x, **B.** magnification x400.

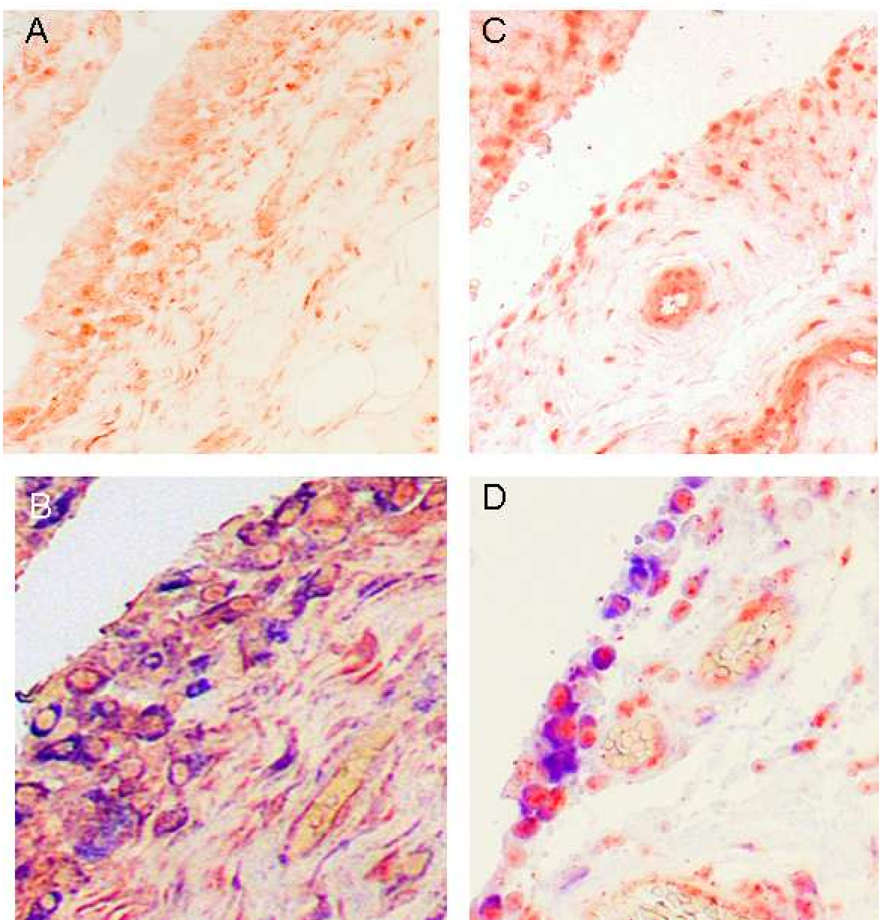


Fig. 4.6 Immunohistochemical detection of galectin-9 in synovial tissue from RA and OA patients – fibroblasts.

Left panel (A-B) shows RA, right panel (C-D) OA synovia. Tissue sections were stained with anti-galectin-9 antibodies (red) (**A**, **C**), with anti-galectin-9 (red) and anti-prolyl hydroxylase (blue) (**B, D**). Original magnification x200.

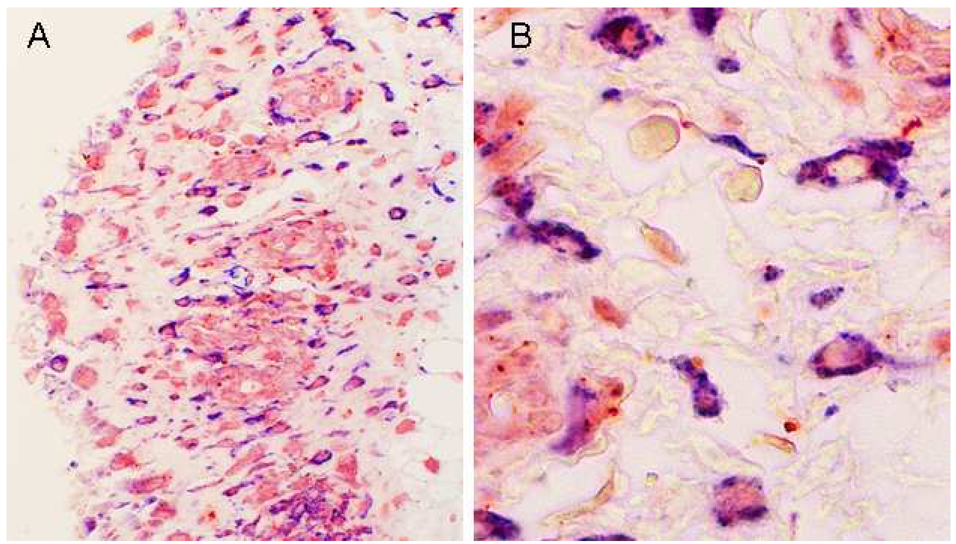


Fig. 4.7 Detection of galectin-9 in RA synovial tissue – macrophages.

Tissue sections were stained with anti-galectin-9 antibodies (red) and anti-CD68 (blue). A. magnification x200, B. magnification x400.

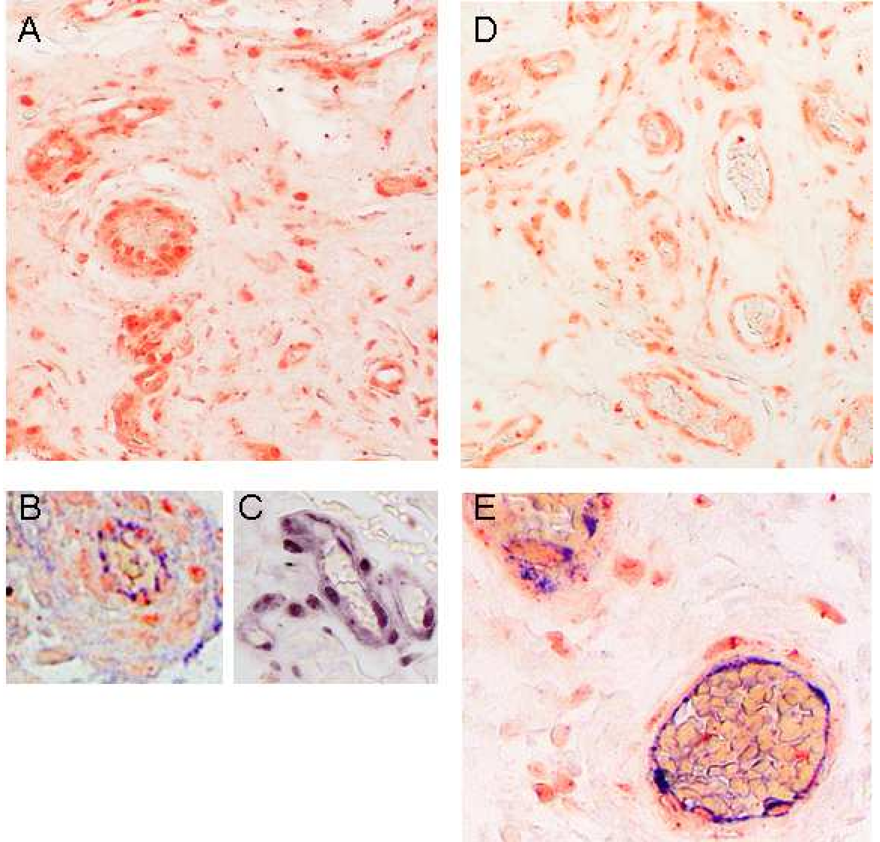


Fig. 4.8 Immunohistochemical detection of galectin-9 in synovial tissue from RA and OA patients – endothelial cells.

Left panel (A-C) shows RA, right panel (D-E) OA synovia. Tissue sections were stained with anti-galectin-9 antibodies (red) (**A**, **D**), with anti-galectin-9 (red) and anti-von Willebrand factor (blue) (**B, E**). Fig. C shows galectin-9 staining (purple). Original magnification A, B, D x200, C and E x400.

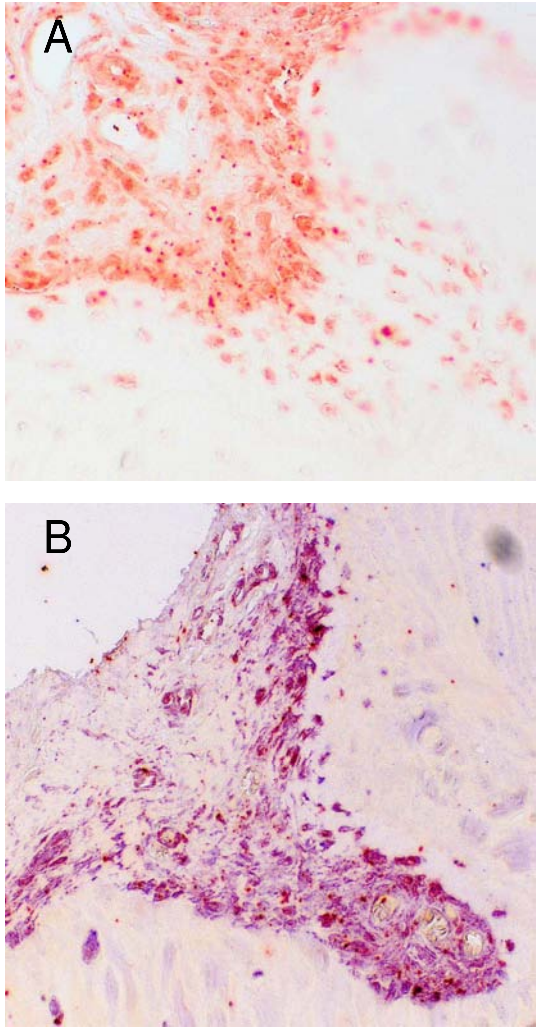


Fig. 4.9 Galectin-9 in RA fibroblasts at sites of invasion.

RA tissue sections of synovium invading cartilage and bone were stained with anti-galectin-9 (red) (**A**) or with anti-galectin-9 (red) and anti-prolyl hydroxylase (blue) antibodies (**B**). Magnification x200.

4.4 Regulation of expression of galectin-3 and galectin-9

Having established the profile of galectin expression in resting fibroblasts it was investigated how they might be regulated in pathological conditions. Possible factors affecting galectin expression in the rheumatoid joint microenvironment could be inflammatory cytokines or ligands for pattern recognition receptors such as Toll-like receptors. It was also hypothesised that epigenetic mechanisms may be involved in regulation of galectin expression in RA fibroblasts, as the pattern of expression is maintained through many passages in culture.

4.4.1 Cytokines

Studies on the regulation of galectins in fibroblasts so far showed increased galectin-3 protein in RA synovial fibroblasts after TNFα treatment (Ohshima et al., 2003). It was also reported that IFNγ may induce galectin-9 in lung and dermal fibroblasts (Asakura et al., 2002). In the present study a potential role for other macrophage, stromal and T cell-derived cytokines on galectin expression by fibroblasts was assessed. IL-1β, TNFα and IL-6 are among the most prominent cytokines found in the synovium (Arend and Gabay, 2006). TNFα is of primary importance in the pathogenesis of RA (Feldmann et al., 1996). Together with IL-1β it is a potent activator of synovial fibroblasts inducing production of inflammatory mediators (cytokines, chemokines, prostaglandins), enzymes and adhesion molecules (McInnes and Schett, 2007; Konttinen et al., 2000). IL-6 mediates some systemic manifestations of the disease and is considered a marker of the disease severity (Nishimoto, 2006). IFNγ was also detected in RA synovium (Simon et al., 1994;

Kotake et al., 1997) although other reports describe low levels of this cytokine in established disease (Firestein and Zvaifler, 1987). IFNγ antagonizes some of the actions of TNFα such as induced proliferation or MMP production (Alvaro-Gracia et al., 1990) by synovial fibroblasts. Presence of IL-17 and its role in arthritis gained much interest in the last few years and has already been discussed. IL-4 is a Th2-type cytokine with mostly anti-inflammatory properties (Arend and Gabay, 2006; Horsfall et al., 1997). It was detected at early stages of RA (Raza et al., 2005a) and in certain histological types of synovitis (i.e. granulomatous, (Klimiuk et al., 1997)). Similarly to interferons, IL-4 decreases collagen synthesis by fibroblasts (Konttinen et al., 2000). It also regulates inflammatory mediators production (Dechanet et al., 1995b). TGFβ is another pleiotropic cytokine associated with rheumatoid arthritis (Miossec et al., 1990). It is produced by various stromal cells and modulates fibroblast recruitment and proliferation as well as production of chemokines, enzymes and matrix components (Konttinen et al., 2000).

To assess the role of these inflammatory cytokines on the expression of galectins, matched lines of synovial, bone marrow and skin fibroblasts derived from RA patients were stimulated for 24h with the following cytokines: IL-1β, IL-6, IL-17, IL-4, IFNγ, TNF-α or TGFβ. Galectin-3 mRNA levels in synovial fibroblasts were not affected by cytokine treatments (Fig. 4.10 A). Only fibroblasts derived from skin up-regulated galectin-3 after exposure to IL-4. A similar tendency was seen with IL-6 and IL-17 treatments but the response was more variable among individual samples and therefore statistically not significant. Interestingly, bone marrow fibroblasts down-regulated galectin-3 after IFNγ or TNFα treatment (Fig. 4.10 A).

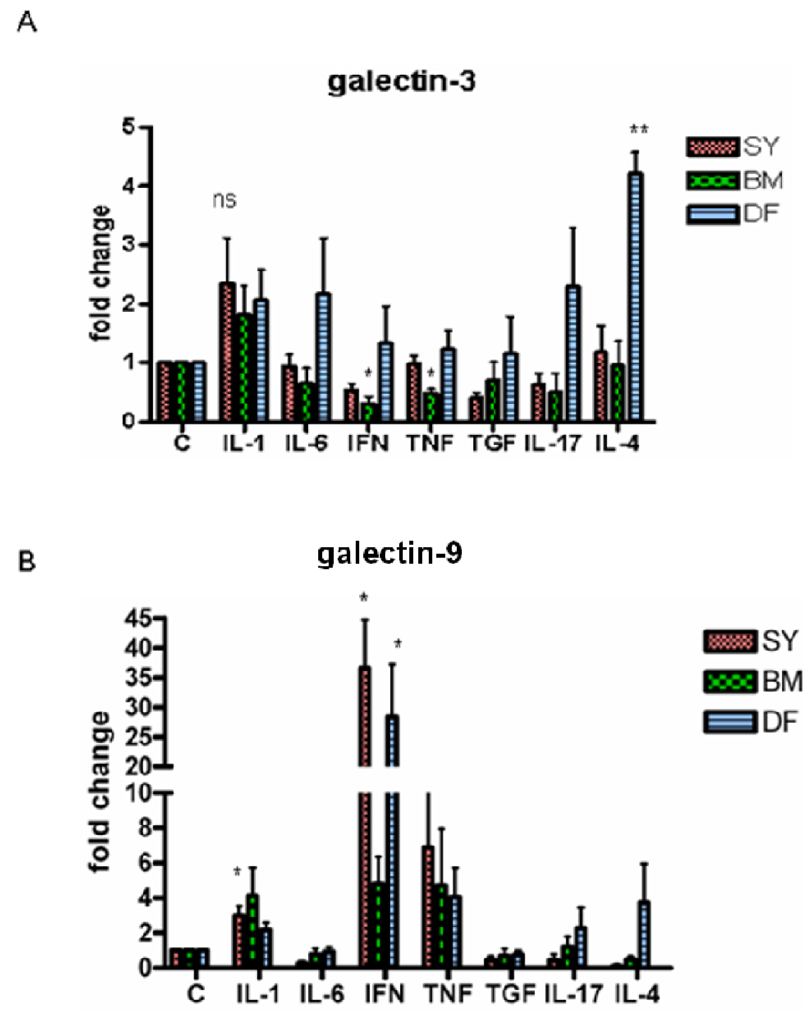


Fig. 4.10 Effect of cytokine stimulation on expression of galectin-3 and -9 mRNA.

Synovial (SY), bone marrow (BM) and skin (DF) fibroblasts were treated for 24 h with 10ng/ml of indicated cytokines. Galectin mRNA expression was quantified by real-time PCR and represented as fold change of expression by non-treated cells (C-control). Bars represent mean values from 5-6 synovial and skin cell lines and 3-4 bone marrow lines. **A.** Changes in galectin-3 expression **B.** Changes in galectin-9 expression. * $p<0.05$, ** $p<0.01$ as compared to expression in control cells from the same site.

Increased levels of galectin-9 transcript were observed in synovial fibroblasts stimulated with IL-1β and TNFα (Fig. 4.10 B). However, because responses to the latter varied considerably among individual samples they did not reach significance with such low numbers of samples. IL-1β, on the other hand, had a modest but much more consistent effect on galectin-9 transcription (2-3 fold increase). However, the most pronounced effect on expression of galectin-9 was observed upon IFNγ treatment (Fig. 4.10 B). This induction at the mRNA level was followed by increase of galectin-9 protein (Fig. 4.11). Simultaneous addition of IFNγ-neutralising antibody (5μg/ml) with IFNγ treatment resulted in inhibition of this effect (Fig. 4.11). Since the estimated molecular weight of galectin-9 short, medium and long isoform is 34.5 kDa, 35.9 kDa and 39.5 kDa, respectively (Chabot et al., 2002), the observed isoforms most likely correspond to medium and long forms.

To elucidate the mechanism underlying IFNγ-induced galectin-9 upregulation
the signalling pathways involved were studied further. IFNγ can signal by a classical pathway involving receptor-associated tyrosine kinases of the Janus family (JAK1, JAK2) and transcription factor STAT (signal transducer and activator of transcription) but also activate alternative STAT-independent ways involving Akt kinase or Rap-1 (Kaur et al., 2008; Alsayed et al., 2000). STAT molecules are recruited to the activated receptor and phosphorylated in order to transduce the signal. p38 MAP kinase performs additional phosphorylation of STAT1 molecule which enhances its transcriptional acitivity (Schindler et al., 2007; Platanias, 2003).

To assess the activation of STAT1 transcription factor following IFNγ stimulation synovial fibroblasts were treated with 10ng/ml recombinant cytokine for various periods of time and then cell lysates prepared and analysed by Western blotting.

Phosphorylation of STAT1 could be seen as soon as 5 min. after addition of IFNγ and steadily increased over time (Fig. 4.12). Additionally blots were probed with an antibody for phosphorylated form of p38 MAPK. Activation of p38 was greatest after 15 min. and then decreased thereafter (Fig. 4.12).

Phosphorylated STAT1 translocates to the nucleus where it can bind to promoters of IFN-inducible genes (Schindler et al., 2007). To verify whether the activated transcription factor changed its subcellular localization in fibroblasts after treatment with IFNγ, cells were stimulated for 15 min. then fixed, permeabilized, stained with anti-STAT1 antibody and analyzed by confocal microscopy. As seen in Fig. 4.13A untreated cells show cytoplasmic STAT1 staining, but in IFNγ-stimulated cells STAT1 co-localized with the red nuclear staining indicating an effective translocation into the nucleus (Fig. 4.13B). Because STAT phosphorylation can occur in response to various growth factors present in serum, in both experiments cells were grown in low-serum medium (0.5% FCS) for 3 days prior to treatment.

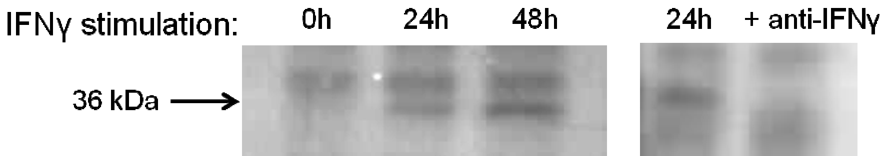


Fig. 4.11 Galectin-9 protein in synovial fibroblasts after IFNγ treatment.

RA synovial fibroblasts were treated with 10ng/ml IFNγ for 24h, 48h or with IFNγ and 5μg/ml neutralising anti- IFNγ antibody for 24h. Cell lysates were prepared and analysed by Western blot. Two bands correspond to galectin-9 isoforms of different length.

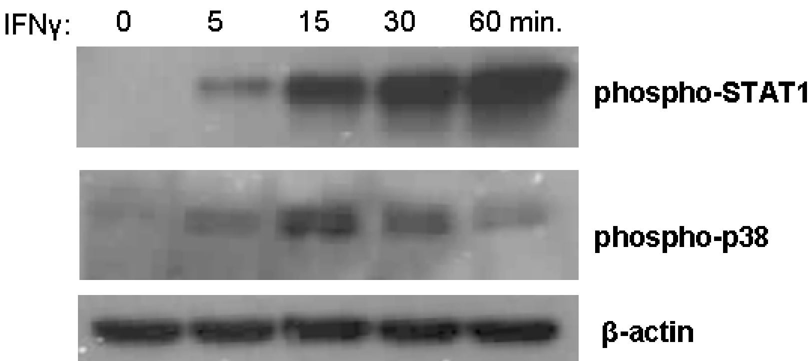


Fig. 4.12 Activation of STAT and p38 MAPK in synovial fibroblasts stimulated with IFNγ.

RA synovial fibroblasts were treated with 10ng/ml IFNγ for 5, 15, 30, 60 min. Cell lysates were prepared for Western blot. Phosphorylation of STAT1 and p38 was assessed by probing with phospho-specific antibodies. β-actin was used as loading control.

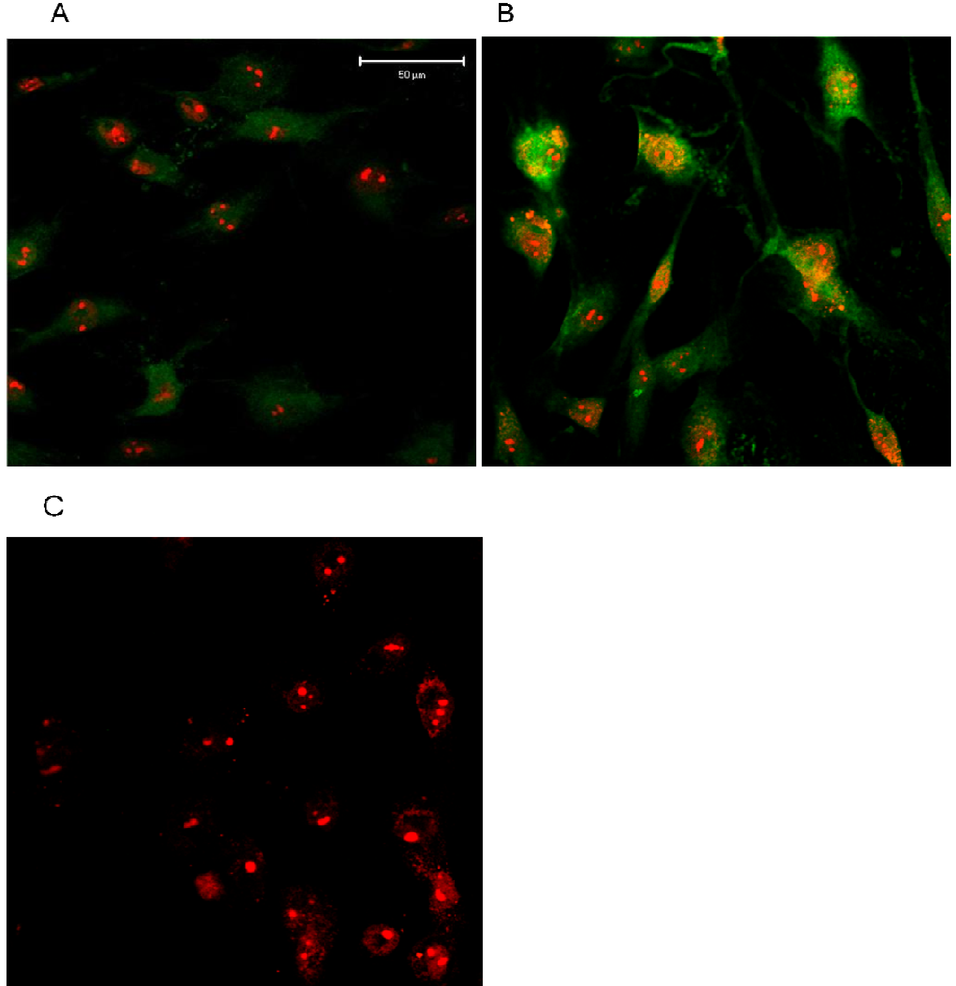


Fig. 4.13 Translocation of STAT in synovial fibroblasts stimulated with IFNγ.

RA synovial fibroblasts were stained with anti-STAT1 antibody (green), nuclei were counterstained with propidium iodide (red). Bar indicates a 50µm scale. **A.** untreated cells **B.** cells treated with 100ng/ml IFNγ for 15min. **C.** negative control (primary antibody omitted)

4.4.2 Toll-like receptors ligands

Toll-like receptors constitute an important part of innate immune system and allow for early detection of infectious agents. They recognize pathogen-associated molecular patterns (PAMPs) such as conserved bacterial cell wall structures (lipopolysaccharides, lipopeptides, proteoglycan), bacterial unmethylated DNA or viral RNA (Akira et al., 2001). Engagement of Toll-like receptors leads to activation of antigen-presenting cells expressing them and to presentation of microbial antigens to cells of the adaptive immune system (Schnare et al., 2001). It was demonstrated that stimulation of immune responses by bacterial TLR ligands can induce arthritis in animal models (Joosten et al., 2003).

While no specific infectious organism has been isolated from joints of RA patients, bacterial products were often noted in rheumatoid patients and those with other arthritides (Wilkinson et al., 1999; Siala et al., 2009). This suggests that such agents may contribute to inflammatory reaction in the joint by acting as adjuvants in the host response.

However, it was also suggested that TLRs bind some endogenous ligands and can cause activation of immune response in the absence of infection. They are involved in response to injury, repair of damaged tissues or clearance of cell debris (Marshak-Rothstein, 2006). Certain heat shock proteins, fibronectin and HMGB (high mobility group box 1 protein) released from damaged cells or breakdown products from extracellular matrix such as hyaluronate may serve as ligands for TLR2 and/or 4 (Ohashi et al., 2000; Roelofs et al., 2006; Park et al., 2006; Termeer et al., 2002). Moreover, double or single stranded RNA from dead cells can be detected by TLR3 and TLR7/8, respectively (Kariko et al., 2004; Zare et al., 2004).

RA synovial fibroblasts were shown to express TLR2 and TLR4, both in culture and in synovial tissue (Seibl et al., 2003; Radstake et al., 2004). Expression of TLR 3 which recognizes double stranded RNA was also reported in RA synovium (Brentano et al., 2005; Ospelt et al., 2008). Stimulation of these receptors with specific ligands (peptidoglycan or lipopeptide for TLR2) led to increased secretion of inflammatory mediators such as cytokines (IL-6), chemokines (CXCL8, CCL5, CCL8, CXCL6, CXCL2), cyclooxygenase and matrix metalloproteinases (MMP-1, -3, -13) (Pierer et al., 2004; Kyburz et al., 2003; Ospelt et al., 2008). Similar responses were observed upon TLR3 activation, namely production of IFNβ, IL-6, CCL5, CXCL10, MMP-3 and -13 (Brentano et al., 2005).

To address the question whether activation of toll-like receptors can affect expression of galectins, RA synovial fibroblasts were stimulated with specific ligands. Bacterial lipopeptide (bLP) which is a component of Gram-positive cell wall was used as ligand for TLR2. Polyinosinic–polycytidylic acid (poly I:C), an authentic double-stranded RNA was used to trigger TLR3 and lipopolysaccharide (LPS), an integral component of the outer membranes of Gram-negative bacteria for TLR4. IL-1β and TNFα treatments were also used for comparison with the previous results. RNA expression of galectins was measured by real-time PCR. While galectin-3 mRNA level was not affected by any of the ligands (Fig. 4.14A), galectin-9 showed marked increase following TLR3 engagement. It was also up-regulated by lipopolysaccharide though to a lesser extent (Fig. 4.14B). IL-1β had a similar effect as in previous experiments, although it was used at a lower concentration (1ng/ml).

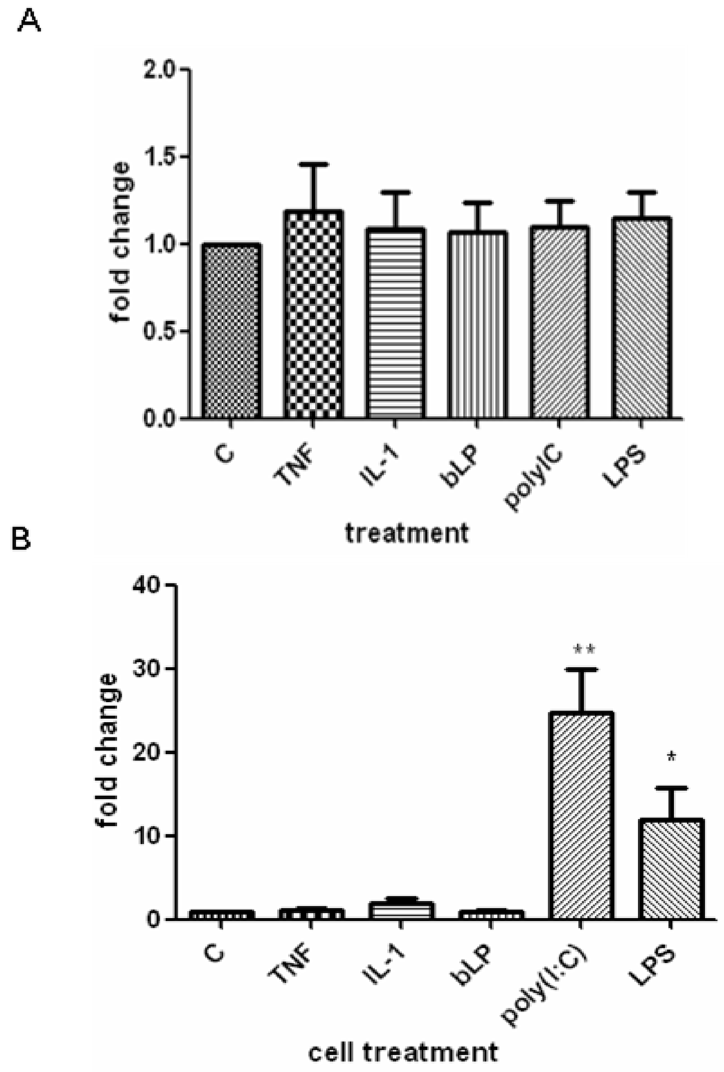


Fig. 4.14 Effect of TLR-ligands on expression of galectin-3 and -9 mRNA.

Synovial fibroblasts were treated with: 10ng/ml TNF, 1ng/ml IL-1β, 300ng/ml bacterial lipoprotein (bLP), 10µg/ml poly(I-C), 100ng/ml lipopolysaccharide (LPS) for 24h. RNA was extracted and reverse-transcribed into cDNA. Galectin levels were measured using specific Taqman probes and normalized with 18S. **A**. Galectin-3 **B.** Galectin-9. Bars represent mean +SEM from 4 experiments, * $p<0.05$, ** $p<0.01$ compared to expression in untreated cells.

4.4.3 Epigenetic factors – DNA methylation

Epigenetic changes define all heritable changes to gene expression which are not encoded by DNA sequence itself. The major epigenetic regulatory mechanisms comprise posttranslational modifications to histones or other chromatin-associated proteins, methylation of DNA and microRNA activity (Karouzakis et al., 2009; Kouzarides, 2007). In mammalian cells methylation is found throughout the genome (Suzuki and Bird, 2008). This contrasts with bacterial genetic material which has a low status of methylation and allows for specific recognition of foreign DNA via TLR9 (Pedersen et al., 2005).

In normal conditions addition of methyl residue to cytosine by methyl transferases (DNMT) prevents binding of transcription factors to DNA molecules and leads to stable transcriptional silencing of a gene (Sanchez-Pernaute et al., 2008; Miranda and Jones, 2007). This mechanism has a fundamental role in embrionic development, X chromosome inactivation, cell differentiation as well as silencing of retroviral elements (Karouzakis et al., 2009; Suzuki and Bird, 2008). Incorrect methylation patterns can result in dedifferetiation of cells and impose new characteristics on them. Indeed, altered DNA methylation of many genes has been associated with transformation of cells into tumour phenotype (Jones and Baylin, 2007). Hypomethylation can also occur in inflammatory diseases (Kim et al., 1996). It is becoming evident that epigenetic processes may influence the development of rheumatic diseases (Strietholt et al., 2008). Recent reports on global genomic hypomethylation in RA synovial fibroblasts suggest that activation of many genes normally silenced by methylation may contribute to the appearance of activated transformed phenotype of these cells (Karouzakis and et al., 2007; Karouzakis et al.,

2008). For example, demethylation of IL-6 promoter could be responsible for the constitutive production of this cytokine (Nile et al., 2008). IL-6 in turn regulates DNA methyltransferase affecting methylation of other genes (Karouzakis et al., 2009).

Promoter regions with a high frequency of CpG dinucleotides, referred to as CpG islands, are indicative of a gene's susceptibility to regulation by methylation. As for galectins, it was demonstrated that DNA methylation is responsible for changes in galectin-1 gene expression during development (Benvenuto et al., 1996) as well as for silenced expression of galectin-7 in nonaggressive lymphoma cells (Moisan et al., 2003). Galectin-3 promoter sequence is also CG rich (74%) (Kadrofske et al., 1998). As depicted in Fig. 4.15 this region can be identified as a CpG island. In contrast, the galectin-9 promoter sequence does not display such properties.

To assess if methylation status can affect expression of galectin-3 and -9 in RA synovial fibroblasts, cells were cultured in the presence of the methylation inhibitor 5-aza-deoxycytidine (0.1 or 1μM). This cytidine analog does not change methylation status of quiescent cells, but inhibits DNA methyltransferase action during cell division causing demethylation in daughter cells. Fibroblasts were seeded at low density and allowed to expand in culture for 7 days in the hypomethylating milieu. At the end of culture the majority of cells would come from new cells divisions and have global DNA hypomethylation. Cell lysates were then prepared and analyzed by Western blotting for quantification of galectin-3 and -9 levels. Though galectin-9 levels did not change, galectin-3 showed an approximately 30% increase after treatment with 1μM 5-aza-deoxycytidine as compared to untreated cells, indicating that its promoter was demethylated and further activated (Fig. 4.16).

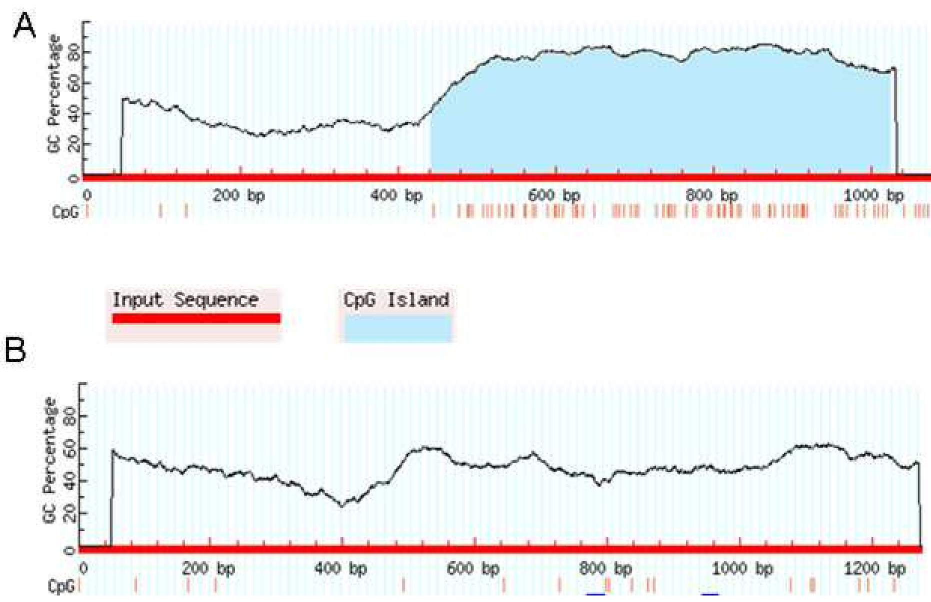


Fig. 4.15 Schematic representation of galectin-3 and galectin-9 promoters.

Figures show results of methylation sites prediction from MethPrimer website (http://www.urogene.org/methprimer/index1.html). Galectin-3 (A) and galectin-9 (B) promoter sequences were taken from Gene Bank. Red ticks show CpG dinucleotides in the DNA sequence, CpG island shaded in blue.

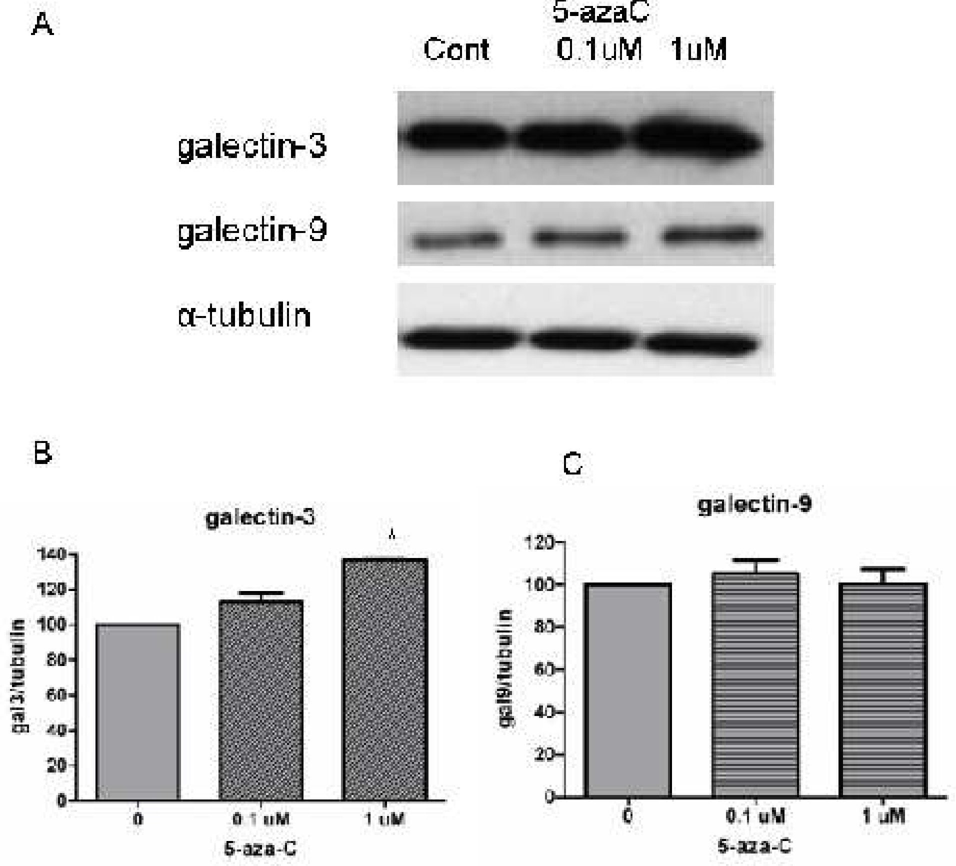


Fig. 4.16 Effect of DNA demethylation on expression of galectin-3 and -9 protein.

Synovial fibroblasts were treated with 0.1 or 1 μM 5-aza-2'-deoxycytidine (5-azaC) for 7 days. Cell lysates were prepared and analysed by Western blotting.

A. Representative blots showing galectin-3, -9 and loading control (α-tubulin).

B and **C.** Quantification of Western blot results. Protein levels expressed as % of control (untreated) cells. Bars represent mean+SD from 4 experiments.*$p<0.05$

4.5 DISCUSSION

Galectin expression profile

This chapter set out to examine the profile of expression of a broad range of galectins and to see if there were site-specific differences among fibroblasts of different anatomical origin. The main focus was on synovium, bone marrow and skin fibroblasts from RA patients, but these were also compared to 4 other primary fibroblast lines (from lung, spleen, tonsil and thyroid). These fibroblasts were assumed to be normal although there is a possibility that the tonsil fibroblasts might have been from inflamed tissue. Due to the lack of complete information on the condition of individuals they were taken from, it was not possible to rule this out. The advantage of using matched cell lines from synovium, bone marrow and skin of the same RA patients is the elimination of genetic variations or possible differences arising from various disease courses and types of treatment. The individual patients' characteristics (disease severity, clinical parmeters, treatment history) may affect the phenotypes and behaviour of fibroblasts obtained from these individuals and account for a wide range of expression levels or responses of cells observed in many experiments. Lack of clinical data to correlate with particular cell lines made interpretation of the results more difficult.

In all fibroblast subsets tested the expression of ubiquitous galectins -1 and -8 was confirmed but without any site-specific differences. These galectins are known to be expressed in a wide range of tissues (Chiariotti et al., 2004) and so the data gained here confirms that reported in the literature. With regard to cells present in the rheumatoid joint, the expression of galectin-1 was previously shown for macrophages, T and B cells (Blaser et al., 1998, Zuniga et al., 2001, Rabinovich et

al., 1998) and in the lining of RA synovium (Ohshima et al., 2003). Galectin-8 has not been reported specifically in fibroblasts although the expression was found in multiple organs (Table 1.1). This galectin is thought to mainly mediate cell adhesion so may have a role in the structural functions provided by fibroblasts. Only one report indicates of a role for galectin-8 in rheumatoid arthritis. It was shown to be a ligand for CD44 expressed on synovial fluid cells and can induce their apoptosis (Eshkar Sebban et al., 2007). The authors suggest that binding of galectin-8 in complexes with CD44 and fibrinogen can cause its sequestration and exacerbation of an inflammatory state.

Galectin-4, on the other hand, seemed to be expressed differently in RA vs. normal fibroblasts. This lectin has been reported as connected with the gastro-intestinal tract (Chiarcotti et al., 2004) but its expression can also be induced in tumours from other tissues (Huflejt and Leffler, 2004). Therefore, its presence in the RA patient tissues was interesting. The expansion of the pannus tissue in the rheumatoid joint has been compared to a transformed tissue and galectin-4 expression may reflect the proliferative state of the synovial stromal tissue. However, it does not explain galectin-4 presence in fibroblasts from the skin, which should not be affected by the inflammatory process.

Another galectin that was found to differentiate synovial fibroblasts from the others was galectin-12. Its transcript has previously been reported in adipose tissue, heart, pancreas, spleen, thymus, and peripheral leukocytes (Yang et al., 2001). Moreover, galectin-12 is up-regulated in cells synchronized at the G1 phase or the G1/S boundary of the cell cycle (Wang et al., 2004). There was no data on its presence in fibroblasts. The function of this galectin was documented as a cell cycle regulator

able to arrest its progression at the G_1 phase and induce cell apoptosis (Yang et al., 2001; Hotta et al., 2001). Finding that galectin-12 may be present in RA synovial fibroblasts but not those from skin may reflect greater proliferative activity of these cells or induction of a regulatory mechanism to suppress it. Lack of expression of galectins 2, -7 and -10 in resting fibroblasts agrees with their restricted cell type-specific expression reported in the literature (Table 1.1).

Galectin-3

Abundant expression of galectin-3 was found in all fibroblasts tested regardless of their site of origin or condition they were taken from (rheumatoid arthritis or osteoarthritis). Studies of synovial tissue in situ revealed also galectin-3 expression by other cell types such as macrophages.

Galectin-3 has been documented previously in macrophages, fibroblasts, chondrocytes and activated T cells (Rabinovich et al., 2002; Perillo et al., 1998). It may be overexpressed in some types of cancers (van den Brule et al., 2004). Increased galectin-3 together with decrease in galectin-1 expression has been associated with inflammation (Andre et al., 1999; Ellerhorst et al., 1999). Indeed, such an altered pattern can be observed in synovial tissue of patients with juvenile idiopathic arthritis (Harjacek et al., 2001). With regard to RA, galectin-3 presence was found previously in cultured synovial fibroblasts and in situ in the synovium but without identification of specific cell types (Ohshima et al., 2003). Here it was confirmed that synovial fibroblasts expressed high levels of galectin-3, however no disease specific differences between cells from RA and OA patients were seen. Because of technical difficulties in obtaining synovial fibroblasts from healthy

individuals only one such cell line was analysed. The results of galectin-3 expression levels were similar to those from RA and OA patients suggesting that the disease process does not result in intracellular accumulation of RNA or protein. Ohshima et al. reported increased levels of galectin-3 in serum and synovial fluid of RA patients compared to OA and healthy controls but also failed to observe differences at the RNA or intracellular protein levels in synovial fibroblasts (Ohshima et al., 2003). This indicates that the regulation of galectin-3 production may occur during translation or secretion of the protein.

It could be argued that in vitro culture conditions influence the expression of galectin-3 and cause all fibroblasts to converge to a similar phenotype. Galectin-3 has a serum response element in its promoter (Kadrofske et al., 1998) and therefore could be induced in cells expanded in standard medium containing 10% fetal calf serum. However, the previous study which used serum-starved cells did not show significant difference in galectin-3 mRNA level in low (0.05%) vs. high (10%) serum (Ohshima et al., 2003).

Another possibility to explain the elevated levels of galectin-3 in RA is that other cells in the synovium apart from synovial fibroblasts contribute to its production. In order to identify them tissue sections of RA and OA synovium were examined by immunohistochemistry. Both in RA and OA fibroblasts showed intense positive staining for galectin-3 protein. Due to higher numbers of cells in the hyperplastic synovial layers, RA tissue samples had more galectin-3. Macrophages were also abundant in RA, but not OA synovium, and therefore may significantly contribute to enhanced galectin-3 production in this disease.

Expression of galectin-3 in synovial fibroblasts can be regulated by external stimuli. This study revealed that epigenetic modifications in galectin-3 promoter may be more important for the regulation of its expression in RA synovial fibroblasts than the action of cytokines or TLR ligands.

Previously it was shown that RA fibroblasts up-regulate galectin-3 upon adhesion to cartilage components (cartilage oligomeric matrix protein, COMP), a process mediated by integrins (Neidhart et al., 2005). Fibroblasts derived from osteoarthritis patients' joints showed less adhesion to this substrate. This observation would suggest that increased galectin-3 in RA synovium may be a secondary effect caused by higher expression of integrins and presence of their ligands (Neidhart et al., 1997) rather than being an intrinsic property of RA synovial fibroblasts.

Other factors potentially influencing galectin expression in rheumatoid joint are inflammatory cytokines. We did not observe significant changes in galectin-3 mRNA in synovial fibroblasts following various treatments, although in some fibroblast cell lines it seemed to be up-regulated by TNFα. It was reported that intracellular galectin-3 protein level may increase upon stimulation with TNFα, even though no change in mRNA level could be detected (Ohshima et al., 2003). Therefore RNA transcription rates do not necessarily reflect changes in protein production and in the case of galectin-3 regulation can occur at other levels. Enhanced production of galectin-3 in skin fibroblasts after treatment with IL-4 is consistent with the recently described role for this lectin in skin inflammation, a typically Th2 driven process (Saegusa et al., 2009).

Since exposure to cytokines did not seem to affect galectin-3 expression in synovial fibroblasts, it was examined whether epigenetic regulatory mechanisms may be

involved. Methylation of galectin-3 promoter was previously shown in pituitary tumours, breast and thyroid carcinoma cell lines (Ruebel et al., 2005) and prostate cancer cells (Ahmed et al., 2007). In all cases methylation correlated with silencing of the gene and lack of galectin-3, whereas cells producing the protein had unmethylated promoters. RA fibroblasts produce galectin-3 constitutively, therefore the promoter in these cells cannot be completely silenced. However, treatment with demethylating agent 5-aza-cytidine resulted in further upregulation of galectin-3 expression which might indicate partial methylation. Alternatively, the observed effect may be indirect, i.e. hypomethylation leads to increased expression of another factor which in turn regulates galectin-3. Given the long time point when the protein measurement was done such possibility cannot be excluded.

Galectin-9

Galectin-9 was found to be expressed at higher levels in synovial fibroblasts from rheumatoid joint than from other sites. Cytokines such as IFNγ or IL-1β and Toll-like receptor ligands may by involved in regulation of its expression.

Galectin-9 is characteristic for some myeloid cells (eosinophils, monocytes, macrophages) but was also identified at certain developmental stages in the thymus and liver (Wada et al., 1997). Expression in fibroblasts was shown earlier by Asakura et al. (2002). During the course of this study an interesting paper by Seki et al. was published. The authors showed expression of galectin-9 in many cells of the RA synovium including vimentin-positive cells, most of which are fibroblasts, endothelial cells and lymphocytes (Seki et al., 2007).

The present study compared galectin-9 expression in synovial tissue of rheumatoid arthritis and osteoarthritis patients. Presence of this galectin was noted in fibroblasts, endothelial cells and macrophages. A remarkable finding was the strong expression of galectin-9 in fibroblasts invading the cartilage and bone. This observation led to further studies on possible roles of galectin-9 in synovial fibroblasts which are described in chapter 5.

Some previous reports did not detect galectin-9 in fibroblasts in physiological conditions but found an up-regulation of this galectin upon stimulation with inflammatory cytokines (Hirashima et al., 2004). However, in contrast to Jurkat cells that secrete galectin-9 after activation with phorbol 12-myristate 13-acetate (PMA) treatment (Chabot et al., 2002) the increased expression of galectin-9 on fibroblasts was not matched with its release measured as eosinophil chemoattractant activity (ECA) (Asakura et al., 2002). The authors therefore suggested that fibroblasts may lack specific protease (MMP) involved in shedding of galectin-9 from the surface.

The present study showed that galectin-9 was expressed in fibroblasts in culture but at a very low level (both mRNA and protein) compared to other galectins, for example galectin-3. Interestingly, fibroblasts of synovial origin had significantly higher levels of galectin-9 transcripts compared to skin-derived fibroblasts from the same individuals. This might reflect either site-specific imprinted phenotypes of fibroblasts or changes acquired in the inflammatory microenvironment. Therefore, it was necessary to examine how pathogenic agents from rheumatoid joint modulate galectin-9 expression. Studies on inflammatory cytokine stimulation revealed that galectin-9 expression in synovial fibroblasts was enhanced after exposure to IL-1β and TNFα. However, response to the latter showed great variability (3 to 15 fold)

among different cell lines and with a low number of samples the effect was not statistically significant. The observed variations might be due to previous treatments (for example anti-TNF agents) received by individual patients.

The greatest change in galectin-9 mRNA production was observed following IFNγ stimulation. A similar effect was seen across all fibroblast lines but the increase in synovial and skin fibroblast was a few times higher compared to bone marrow cells. This finding is in agreement with previous reports on skin (Igawa et al., 2006) and lung fibroblasts (Asakura et al., 2002) where galectin-9 mediated eosinophil attraction and adhesion which was implicated in development of fibrotic disease. Enhanced production was also observed at the protein level and could be inhibited by IFNγ-neutralising antibody. Whether the up-regulation of intracellular galectin-9 in RA synovial fibroblasts is followed by increased release into the extracellular space is not known.

Extracellular galectin-9 was shown to induce apoptosis of activated T helper 1 (Th1) cells by interacting with TIM-3 ligand on their surface (Kashio et al., 2003; Zhu et al., 2005). Th1 cells produce large amounts of IFNγ upon activation. Therefore induction of galectin-9 from fibroblasts would serve as a negative feedback loop to prevent an uncontrolled inflammatory response. In RA joint, however, T cells seem to be defective in terms of cytokine production. They are capable of producing this cytokine in vitro when stimulated (Abreu et al., 2009) but the level of IFNγ in synovial fluids of established RA is much lower than in other Th1-mediated diseases (Firestein et al., 1990; Kotake et al., 1997). Suppressed production of interferon-γ as well as IL-2 may result from the presence of large amounts of IL-10 in synovial tissue and fluid (Arend and Gabay, 2006). Given the fact that IFNγ has

antiproliferative effects on fibroblasts (Elias et al., 1987), can counteract certain TNFα-induced effects (Alvaro-Gracia et al., 1990; Williams et al., 2007) and regulates T cell apoptosis via galectin-9-TIM3 interactions, this cytokine could have a homeostatic role in inflammation. However, its regulatory mechanisms are defective in RA and consequently lead to persistent inflammation (Raza et al., 2005a).

Another interesting finding was the up-regulation of galectin-9 by Toll-like receptor ligands: double stranded RNA and bacterial LPS. In contrast, galectin-3 was not affected by these treatments. Induction of galectin-9 by dsRNA was shown previously in vascular endothelium (Imaizumi et al., 2007). In HUVECs LPS also induced galectin-9 mRNA but not protein indicating that control by post-transcriptional mechanisms is important for the effects in these cells. Galectin protein production in fibroblasts following TLRs stimulation was not tested, so it is not clear whether the effect at RNA level translates into functional changes.

Up-regulation of a protein upon IFNγ and TLR3 stimulation may suggest involvement in antiviral responses but this area with regard to galectins remains unexplored. However, it was shown in an animal model that galectin-9 is induced in LPS-triggered inflammation and has a protective, anti-inflammatory role (Tsuboi et al., 2007).

Although evidence for infectious events in pathogenesis of rheumatoid arthritis is scarce, in some patients bacterial products were identified in diseased joints (Siala et al., 2009). Therefore early induction of inflammatory process by activation of synovial fibroblasts via Toll-like receptors cannot be excluded. More importantly, in the absence of pathogenic material TLRs can provoke an inflammatory response by

recognition of endogenous ligands (Kawane et al., 2006; Marshak-Rothstein and Rifkin, 2007). RNA or intracellular proteins released from dead cells and matrix breakdown products such as hyaluranate, fibronogen can engage TLRs. These products are readily available in inflamed joint (Roelofs et al., 2006; Brentano et al., 2005) and can cause activation of resident fibroblasts and perpetuation of inflammatory process at this site (Sacre et al., 2007; Ospelt et al., 2004a). Moreover, inhibition of TLR activation prevented the development of collagen-induced arthritis (Abdollahi-Roodsaz et al., 2007).

Because of the possible induction of galectin-9 in the synovium by cytokines and TLR ligands its potential role was studied further (chapter 5).

5.0 Role of galectin-9 in RA synovial fibroblast function

5.1 Background

Galectin-9 is well recognized as an eosinophil chemoattractant (Mastumoto, Spitzenberger et al., 2001). It is also known to trigger apoptosis of T cells expressing TIM-3 on their surface (Zhu et al., 2005; van de Weyer et al., 2006). Recently Seki et al. identified significant galectin-9 expression in rheumatoid arthritis synovium. The authors also demonstrated induction of apoptosis in synovial fibroblasts by a mutant form of galectin-9 and reported the ability of the mutant galectin-9 to reduce inflammation in the mouse collagen-induced arthritis model (Seki et al., 2007). Whether the effects of the mutant galectin-9 in vivo extended beyond induction of apoptosis was not determined. Moreover, the role for a naturally occurring galectin-9 in these fibroblasts was not established.

The present study revealed that expression of galectin-9 in RA synovial fibroblasts was higher than in skin samples from the same individuals. In addition, RA synovial fibroblasts from the aggressive pannus tissue displayed strong positive staining for galectin-9. It was hypothesised that expression of this galectin may affect synovial fibroblast functions such as proliferation, apoptosis or production of inflammatory mediators and matrix degrading enzymes. This hypothesis was tested in this chapter using an siRNA approach.

5.2 Effect of galectin-9 silencing on fibroblast apoptosis and production of inflammatory mediators

To elucidate what role intracellular galectin-9 may play in RA synovial fibroblasts, the small interfering RNA (siRNA) technique was used to silence its expression. Fibroblasts grown in culture were transfected with 33 nM siRNA designed to bind to all galectin-9 isoforms or with control RNA sequences which do not bind to any known mRNAs. Expression of galectin-9 mRNA was assessed by real-time PCR at 24, 48 and 72h after transfection. Fig. 5.1A shows that the greatest effect at mRNA level was obtained after 72h (96% inhibition). Galectin-9 protein at this time point was also dramatically reduced (Fig. 5.1B).

Spontaneous and induced apoptosis of RA synovial fibroblasts in culture was assessed by staining the cells with annexin-V-FITC and analysed by flow cytometry. It was found that the rates of both spontaneous apoptosis and that induced by the broad spectrum kinase inhibitor staurosporine were higher in galectin-9 knockdown cells than in controls. Galectin-9 silencing resulted in an increase in apoptosis from 5 to 10% for untreated cells and from 35 to over 60% for staurosporine treated cells (Figure 5.2).

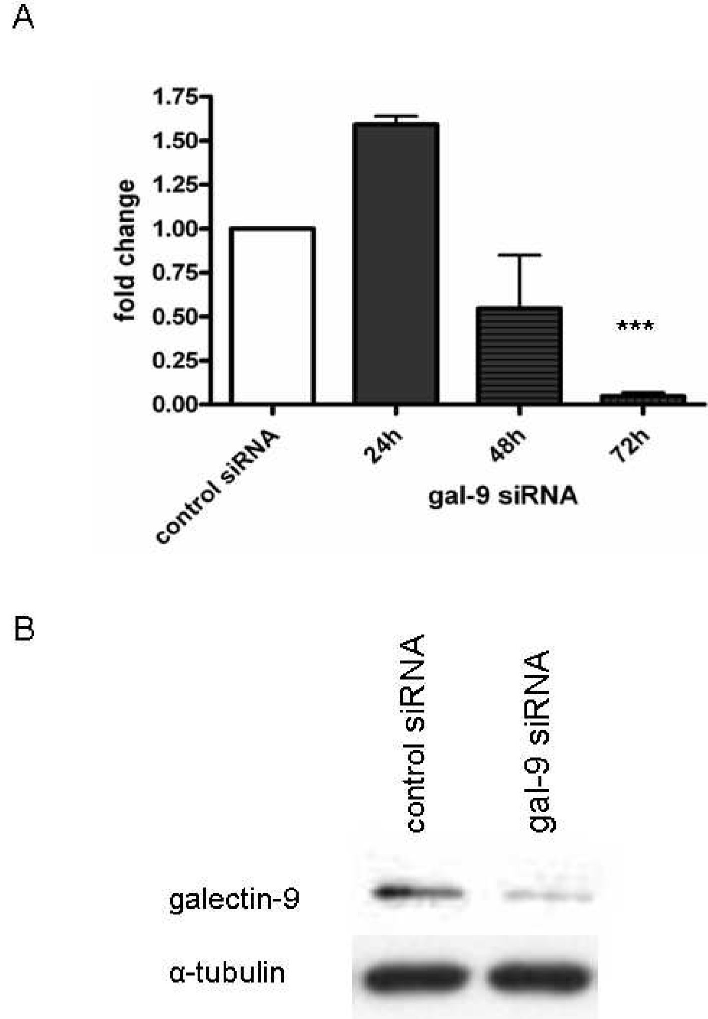


Fig. 5.1 Galectin-9 silencing in RA synovial fibroblasts.

Cells were transfected with galectin-9 siRNA or control siRNA. **A.** Galectin-9 mRNA measured by real-time PCR 24, 48 and 72h after transfection and normalized to 18S. Data are mean +SD from three independent experiments. ***p<0.001, compared to cells transfected with control siRNA. **B**. Galectin-9 protein in synovial fibroblasts 72h after transfection with galectin-9 or control siRNA. α-tubulin was assessed as a loading control.

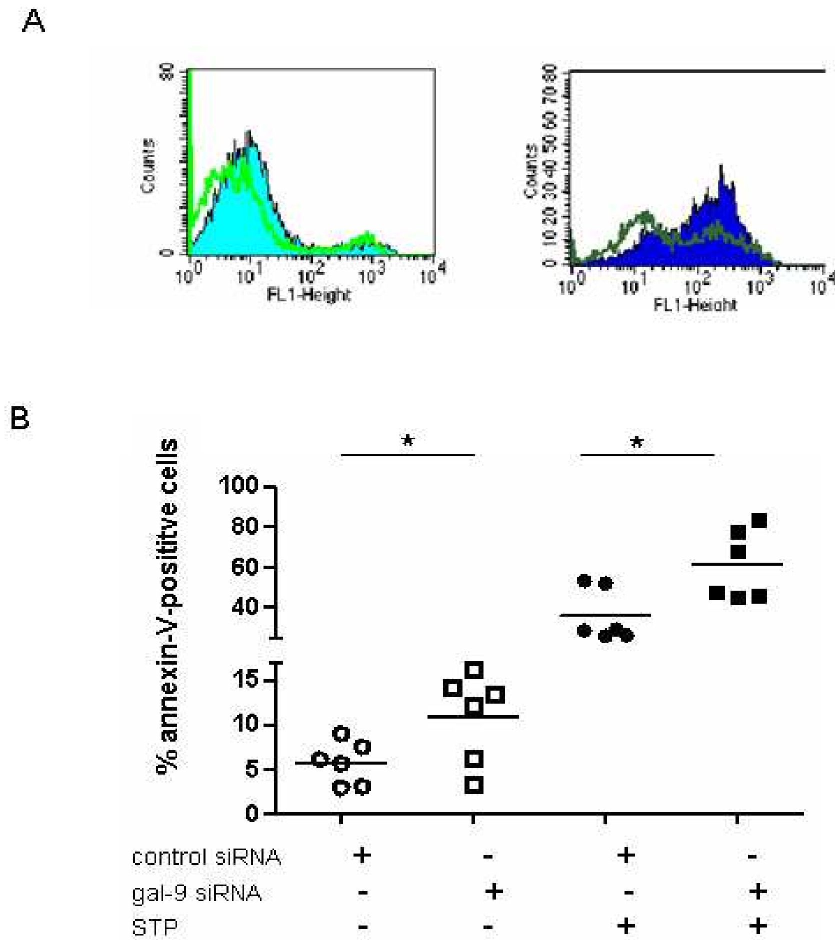


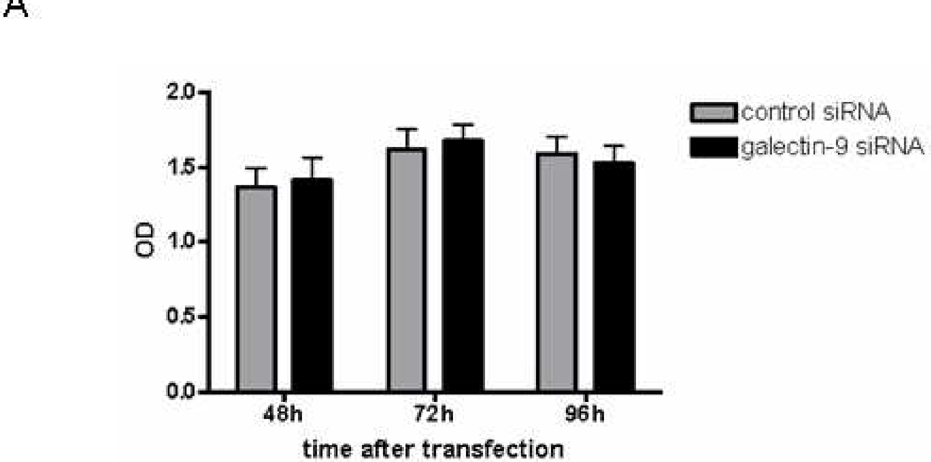
Fig. 5.2 Effect of galectin-9 silencing on synovial fibroblast apoptosis.

Fibroblasts were transfected with galectin-9 or control siRNA. After 72h cells were treated with 5μM staurosporine (STP) for 5h or left untreated. Cells were stained with Annexin-V-FITC and analyzed by flow cytometry to identify apoptotic cells. **A.** Representative histograms showing annexin-V positive cells transfected with control siRNA (left) and gal-9 siRNA (right). Single line-untreated cells, filled-cells treated with STP. **B.** Cumulative data from 6 experiments. Circles-control siRNA transfected cells, squares-gal-9 siRNA transfected cells. * $p<0.05$.

Proliferation of control siRNA and galectin-9 siRNA transfected cells was measured using the XTT Cell Proliferation kit. Cells were transfected with respective siRNAs and either treated with 10ng/ml IL1-β and 10ng/ml TNFα to stimulate proliferation or cultured in medium alone. No significant changes between control and galectin-9 siRNA transfected cells were noted at 2-4 days after transfection (Fig. 5.3).

To determine whether galectin-9 influences the production of mediators of inflammation and cartilage destruction by synovial fibroblasts, supernatants from galectin-9 silenced and control cells were analysed by ELISA for their output of inflammatory cytokines. Although some cell lines displayed a trend towards reduced IL-6 secretion the overall effect was not significant (Fig. 5.4A). There were also no differences in production of the chemokines, IL-8 and CCL5 (Fig. 5.4 C, D), or matrix metalloproteinase-3 (Fig.5.4 B).

A



B

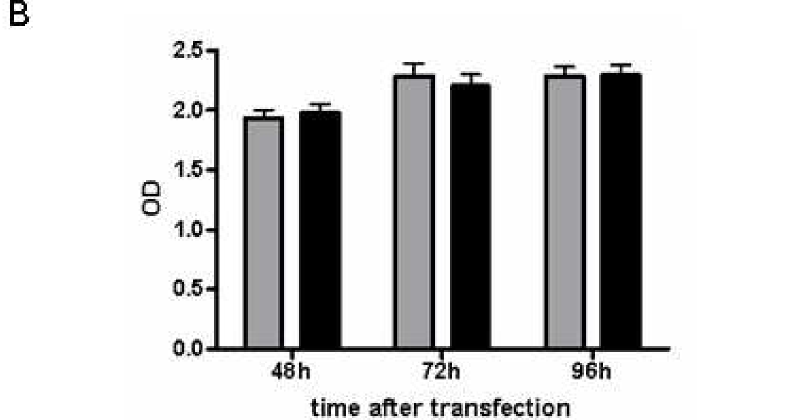


Fig. 5.3 Effect of galectin-9 silencing on synovial fibroblasts proliferation.

Cells were transfected with galectin-9 (black bars) or control siRNA (grey bars). Proliferation was measured by XTT assay at the indicated time points and data are shown as OD values. Data are mean +SEM from 4 experiments run in duplicate. **A.** Unstimulated cells. **B.** Cells stimulated with 10ng/ml IL1-β and 10ng/ml TNFα 24h after transfection.

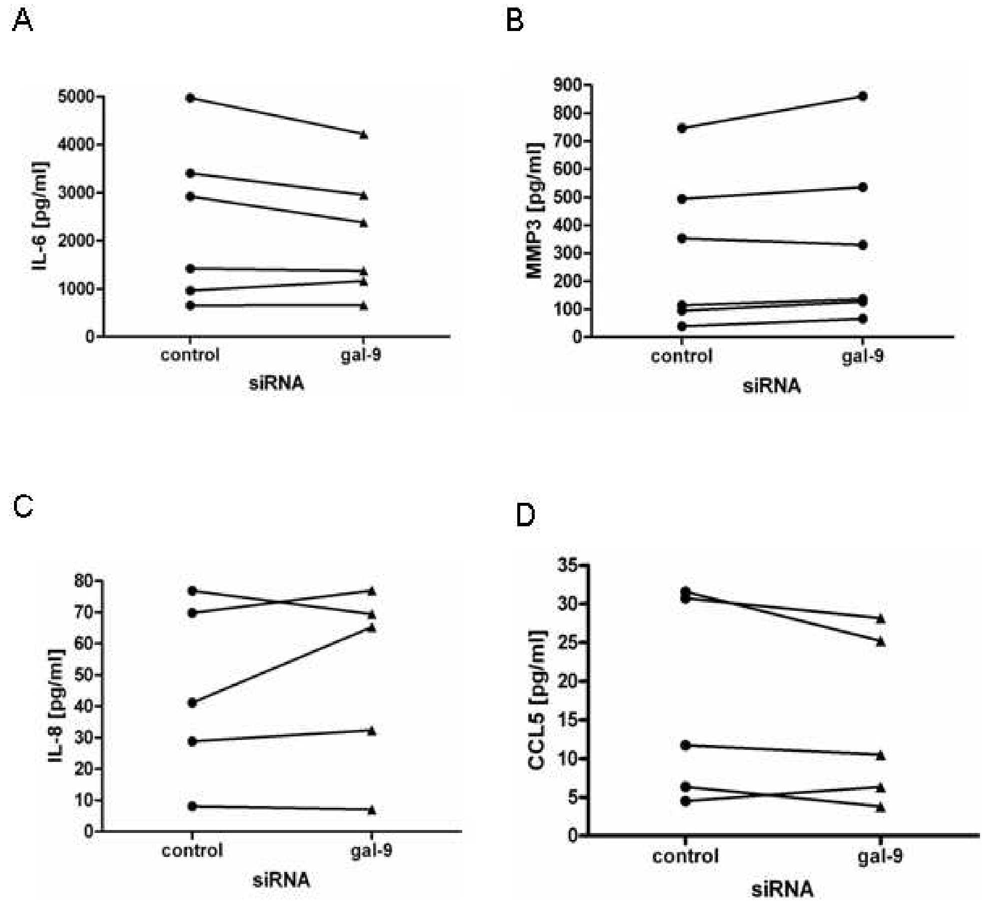


Fig. 5.4 Effect of galectin-9 silencing on production of IL-6, MMP-3, IL-8 and CCL5 by synovial fibroblasts.

Fibroblasts were transfected with galectin-9 or control siRNA. After 72h supernatants were collected and protein measured by ELISA. Lines show results from individual samples. Circles-control siRNA transfected cells, triangles-galectin-9 siRNA transfected cells. A. IL-6 B. MMP-3 C. IL-8 D. CCL5

5.3 DISCUSSION

Galectin-9 silencing in RA synovial fibroblasts resulted in increased rates of apoptosis in these cells. However it did not affect cell proliferation or production of cytokine IL-6, chemokines (IL-8 and CCL5) or MMP-3.

Up to now the only report on galectin-9 in RA synovium was published by Seki et al. (2007). The authors demonstrated that a mutant galectin-9 with deleted linker peptide has a pro-apoptotic effect on synovial fibroblasts. This mutation confers increased stability to the protein which otherwise is susceptible to enzymatic cleavage in vivo (Nishi et al., 2005). Such modified galectin-9 was also effective for HTLV-1-infected T cells (Okudaira et al., 2007). Wild-type molecules (short and medium isoforms) were much less potent in inducing apoptosis of synovial fibroblasts, although other biological activities of all forms were similar (Seki et al., 2007). The above study showed also a beneficial effect of treatment with exogenous galectin-9 mutant in a mouse model of collagen-induced arthritis (CIA), however a role for the naturally occurring protein was not established.

Here it was demonstrated that the intracellular presence of galectin-9 in synovial fibroblasts may confer greater resistance to apoptosis, both spontaneous and induced. In that respect galectin-9 would act similarly to galectin-3. Galectin-3 is capable of triggering apoptosis of T cells and thymocytes when found outside the cell (Fukumori et al., 2003; Stillman et al., 2006) but when it is localized intracellularly it has a protective effect by interacting with anti-apoptotic proteins from the Bcl-2 family (Yang et al., 1996; Matarrese et al., 2000; Akahani et al., 1997). A pro-apoptotic effect of exogenous galectin-9 was previously described for thymocytes, activated T cells and some tumour cells such as melanoma or Burkitt lymphoma

(Makishi et al., 2008; Kageshita et al., 2002). The data reported here suggest that the upregulation of intracellular galectin-9 might induce some degree of resistance to apoptosis in synovial fibroblasts at sites of joint destruction and thus contribute to the expansion of the pannus tissue in RA.

Because higher numbers of apoptotic cells in culture may often be due to increased rates of proliferation, it was examined whether intracellular galectin-9 also affects that process. Both spontaneous and cytokine-induced cell proliferation of RA synovial fibroblasts were similar in control and galectin-9 silenced cells confirming that these processes were independent and not affected by galectin-9. Pro-proliferative role independent of apoptotic processes was documented earlier for galectin-3 in Jurkat T cells (Yang et al., 1996). However, the same galectin overexpressed in prostate cancer cells slowed down their growth (Ellerhorst et al., 1999). Biphasic modulation of proliferation also characterizes extracellular galectin-1 which, depending on its concentration, either promotes cell growth or inhibits it (Adams et al., 1996; Vas et al., 2005). Intracellularly galectin-1 may contribute to cell transformation by association with oncogenic H-Ras (Paz et al., 2001; Elad-Sfadia et al., 2004).

A recent paper by Matsuura et al. implicated intracellular galectin-9 in cytokine production in the monocytic cell line THP-1. Transfection with galectin-9 resulted in activation of promoters for IL-1α, IL-1β and IFNγ in these cells (Matsuura et al., 2009). In the present study we looked for an effect of galectin-9 on the production of cytokines, chemokines and matrix metalloproteinase relevant to RA in synovial

fibroblasts but found no difference in either IL-6, IL-8, CCL5 or MMP-3 output when galectin-9 was suppressed. It would be interesting to investigate the influence of galectin-9 on the expression of other inflammatory mediators, adhesion molecules or molecules of signalling pathways.

In summary, the different roles of galectin-9 depending on its localization seem to be a common feature shared with other galectins. As described previously intracellular galectin-3 interfere with apoptotic pathways rescuing cells from death, while acting from outside of the cell it promotes this process (Yang et al., 1996; Fukumori et al., 2003). Galectin-1 can also exhibit differential effects on cell death (Adams et al., 1996; Paz et al., 2001). To establish which of these functions of galectin-9 predominates in the RA joint it would be crucial to determine whether the protein is released in soluble form and how stable it is in this environment.

6.0 Response of fibroblasts to galectin-3

6.1 Background

Galectins are widely described as regulators of various immune cell subsets, but their influence on stromal cell functions is less well recognized. Galectin-1 was found to be a mitogenic factor for vascular endothelial cells (Hsu and Liu, 2004) but cytostatic for mouse embryonic fibroblasts (Wells and Mallucci, 1992). Galectin-3 similarly induced morphogenesis of endothelial cells and angiogenesis (Nangia-Makker et al., 2000) and also stimulated DNA synthesis and proliferation in lung fibroblasts (Inohara et al., 1998). In the context of synovitis, galectin-3 has been shown to enhance the growth of RA and OA synovial fibroblasts in culture (Ohshima et al., 2003).

Apart from regulating cell growth, galectins can activate fibroblasts to produce various factors. For example galectin-3 has been shown to increase collagen production by liver fibroblasts resulting in organ fibrosis (Henderson et al., 2006) and to stimulate IL-8 secretion from fibroblasts in the colon (Lippert et al., 2007).

Previous work from our own research group has shown that fibroblasts from rheumatoid arthritis patients also respond to stimulation by galectin-1 and -3 with increased production of cytokines, chemokines, growth factors and tissue degrading enzymes (Trebilcock, 2006). For instance, both galectins induced the secretion of IL-6, IL-8 (CXCL8) and MMP-9, although galectin-3 was much more potent in that effect. GM-CSF, IL-1β or CCL4 (MIP-1β) were induced to a similar extent by both lectins. Galectin-3, however, uniquely stimulated the production of TNF-α, CCL3 (MIP-1α), CCL5 (RANTES).

Because of the abundant expression of galectin-3 found in RA synovium, the aim of this chapter was to investigate its potential pathogenic role in rheumatoid arthritis, focussing on its modulation of fibroblasts function. This study also looked at site-specific differences in response to galectin-3 in fibroblasts from RA synovium and skin.

6.2 Differential production of cytokines and chemokines from fibroblasts stimulated by galectin-3

To assess the differential production of inflammatory mediators in response to galectin-3 matched samples of fibroblasts derived from synovium and skin of RA patients were treated with galectin-3 for 24h and the supernatants analysed by ELISA for secretion of proteins. Since the physiological levels of galectin-3 were reported at around 20 μg/ml (Lippert et al., 2007), the concentration of 10 μg/ml was chosen for these studies. It was confirmed that synovial fibroblasts respond to galectin-3 treatment with increased secretion of IL-6, CCL5 and CCL2 (Fig. 6.1). Interestingly fibroblasts from skin in general up-regulated only the production of IL-6 but did not increase secretion of chemokines (Fig. 6.1 B and C).

IL-6 production in synovial and skin fibroblasts as well as CCL5 production in synovial cells was dose-dependent (Fig. 6.2). Although skin fibroblasts did not respond by increasing chemokine production as a rule, increased production of CCL5 was observed in one skin fibroblast sample, but only at high concentrations of galectin-3 (>30μg/ml; Fig. 6.2D).

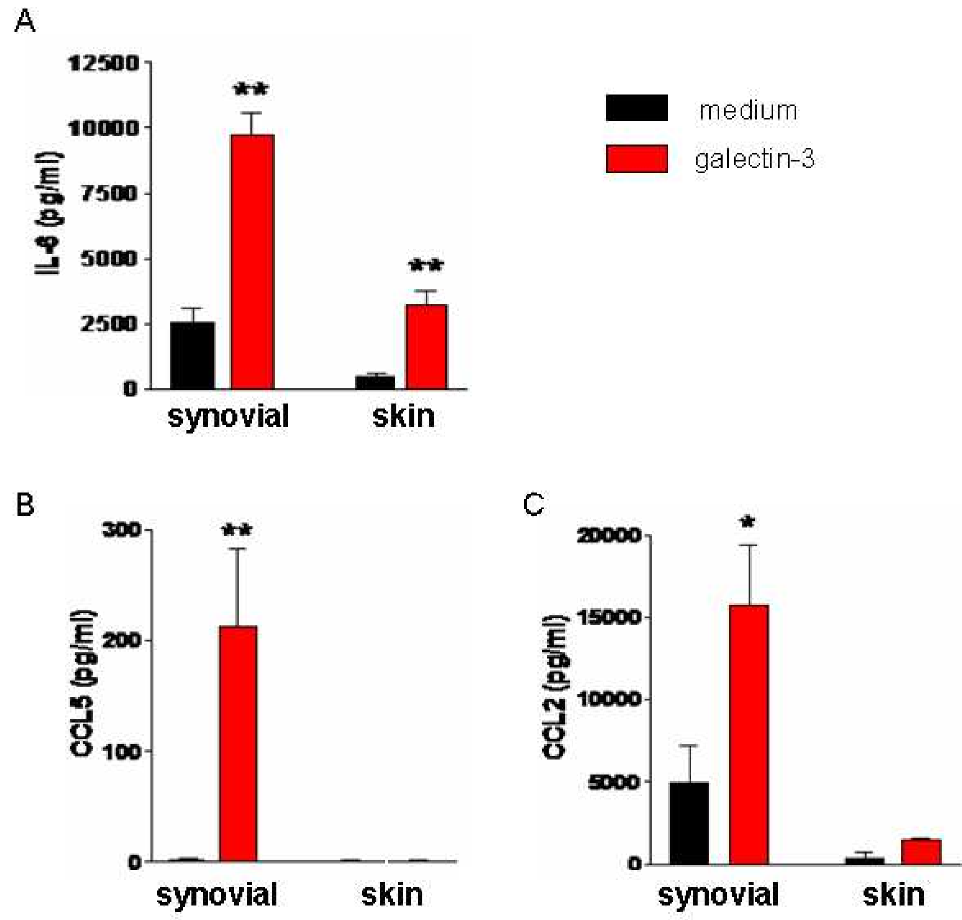


Fig. 6.1 Galectin-induced production of cytokines from fibroblasts.

Matched synovial and skin fibroblasts from RA patients were stimulated with 10 μg/ml galectin-3 for 24h. The level of cytokines (A) IL-6, (B) CCL5 and (C) CCL2, was measured in the supernatant by ELISA. Bars show mean ± SEM from 5 duplicate samples. *$p<0.05$, ** $p<0.01$ compared to production by untreated cells.

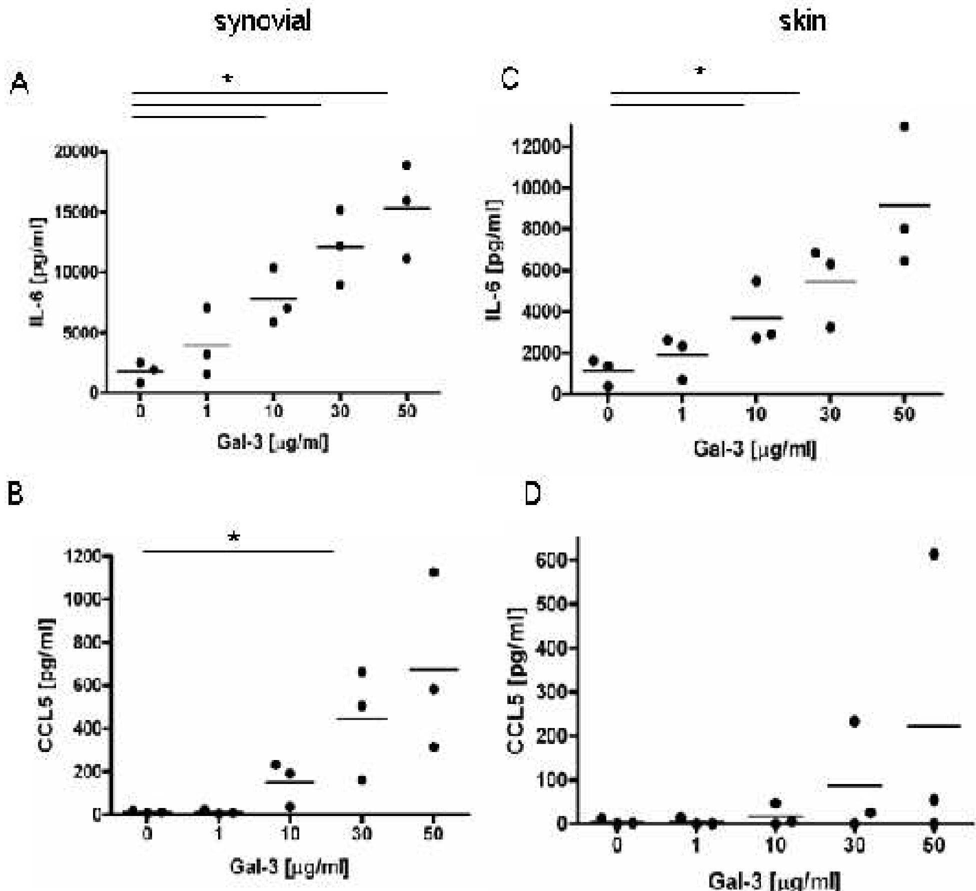


Fig. 6.2 Galectin-3 - induced production of IL-6 and CCL5 is concentration dependent.

Synovial and skin fibroblasts were treated with various doses as shown of galectin-3 for 24h. The level of each cytokine was measured in the supernatant by ELISA. Left panel shows synovial fibroblasts, right panel skin fibroblasts. Bars indicate the mean value and each point is the average of duplicates in individual cell lines. *p<0.05

6.3 Galectin-3-induced IL-6, CCL5 and CCL2 production is not mediated via autocrine TNFα

As mentioned previously, one of the factors induced by galectin-3 in synovial fibroblasts was TNF-α (Trebilcock, 2006). This cytokine is considered to be at the top of the inflammatory cytokine cascade which results in production of IL-6 and other cytokines and chemokines. Hence, there was a possibility that the increase in the production of cytokines and chemokines might be a secondary effect caused by autocrine stimulation by TNF-α. To check if this was the case, 5μg/ml TNF-neutralizing antibody was added to the culture 30 minutes prior to cell activation with galectin-3. 10 ng/ml TNF-α was used as a positive control. There was a complete blockade of the actions of exogenously added TNF-α by the blocking antibody, but no significant effect on either IL-6, CCL5 or CCL2 secretion from galectin-3 stimulated fibroblasts (Fig. 6.3). It appears that increased production of IL-6 and chemokines (CCL5, CCL2) induced by galectin-3 is independent of the presence of TNF-α in fibroblast cultures.

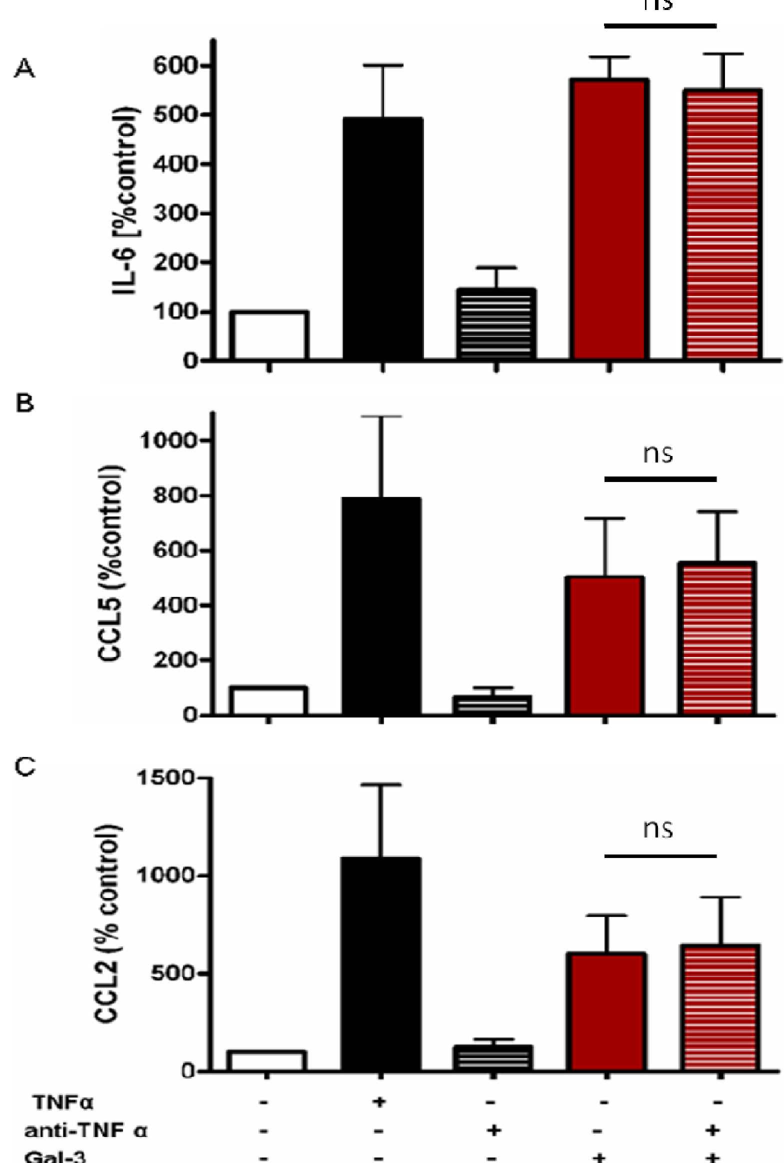


Fig. 6.3 Effect of neutralizing TNFα on galectin-3 induced IL-6, CCL2 and CCL2 production. Neutralizing anti-TNFα antibody was added to synovial fibroblast cultures at 5 μg/ml 30 min prior to stimulation with 10 ng/ml TNF-α or 10 μg/ml galectin-3. Supernatants were collected after 24h and IL-6, CCL5 and CCL2 measured by ELISA. Data are expressed as % of control (either +TNF or + galectin 3) and are the mean + SEM from four independent experiments.

6.4 Signaling pathways induced in fibroblasts by galectin-3

It was next decided to determine the pathways involved in intracellular galectin-3 signalling leading to the differential responses described above. IL-6 and CCL5 were chosen as examples of induced cytokine and chemokines, respectively. To investigate which pathways may be activated a number of inhibitors against different kinases involved in pro-inflammatory pathways were applied to galectin-3 stimulated cells (Fig. 6.4). The production of IL-6 and CCL5 was used as a readout.

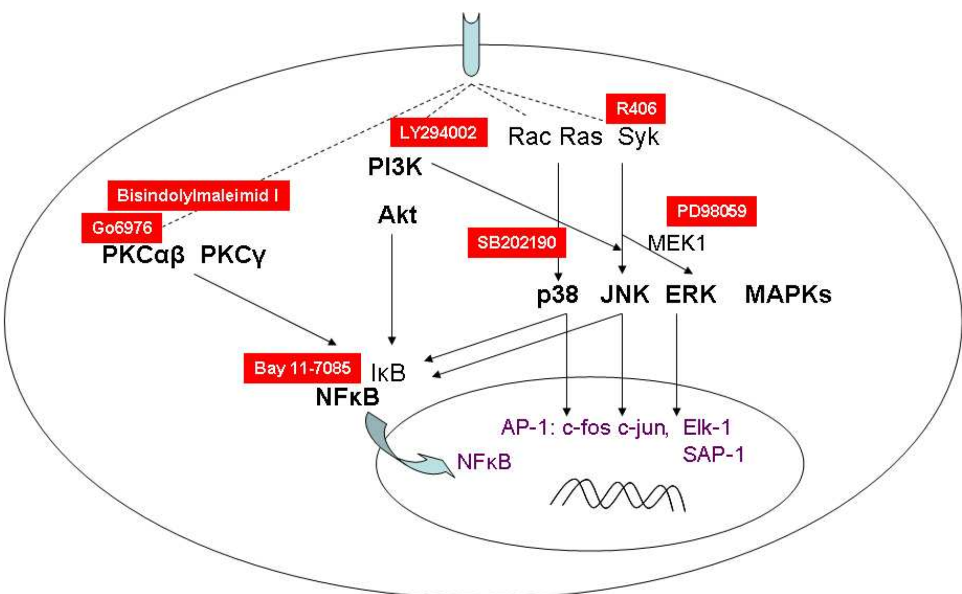


Fig. 6.4 Diagram of potential galectin-3 signalling pathways and inhibitors used to examine them.

The figure depicts selected protein kinases that may be involved in transducing signals leading to cytokine/chemokine production in fibroblasts. Arrows show activation. Inhibitors are in red, putative receptors are shown in blue.

Fibroblasts were treated for 1h with one of the following inhibitors: 5 μM Bay11-7085, 10 μM LY294002, 1 μM bisindolylmaleimide, 20 nM Gö6976, 10 μM SB20219, 10 μM R406 or 10 μM PD98059, before stimulation with 10 μg/ml galectin-3. After 24h of culture supernatants were collected and IL-6 and CCL5 levels measured by ELISA. It was found that in synovial fibroblasts IL-6 production was reduced by blocking the MAPK pathway, with inhibition of p38MAPK (inhibited by SB202190), Syk/JNK (inhibited by R406) and ERK (inhibited via blockade of MEK by PD98059). Application of these inhibitors resulted in at least 50% inhibition of IL-6 production (Fig. 6.5 A). Skin fibroblasts responded to p38 and Syk/JNK inhibition (SB202190, R406) in a similar way, but the MEK1/ERK inhibitor (PD98059) had no effect on IL-6 production (Fig. 6.5B). In both cell types interfering with NFκB activation by Bay11-7085 also significantly reduced IL-6 secretion. (Fig. 6.5C) by 65 % in synovial and 80% in skin fibroblasts.

In contrast, CCL5 production in synovial cells was affected by inhibitors of PI3K and NFκB, with production reduced by 65% and 40%, respectively (Fig. 6.6), but MAPK inhibitors were without effect. Skin fibroblasts did not produce detectable amounts of CCL5 after treatment with 10 μg/ml galectin-3. The PKC pathway did not appear to be required for the actions of galectin-3 as both bisindolylmaleimide and Go6976 were without effect. The inhibitors had no significant effect on cell numbers or morphology so reduced protein production due to toxicity was excluded.

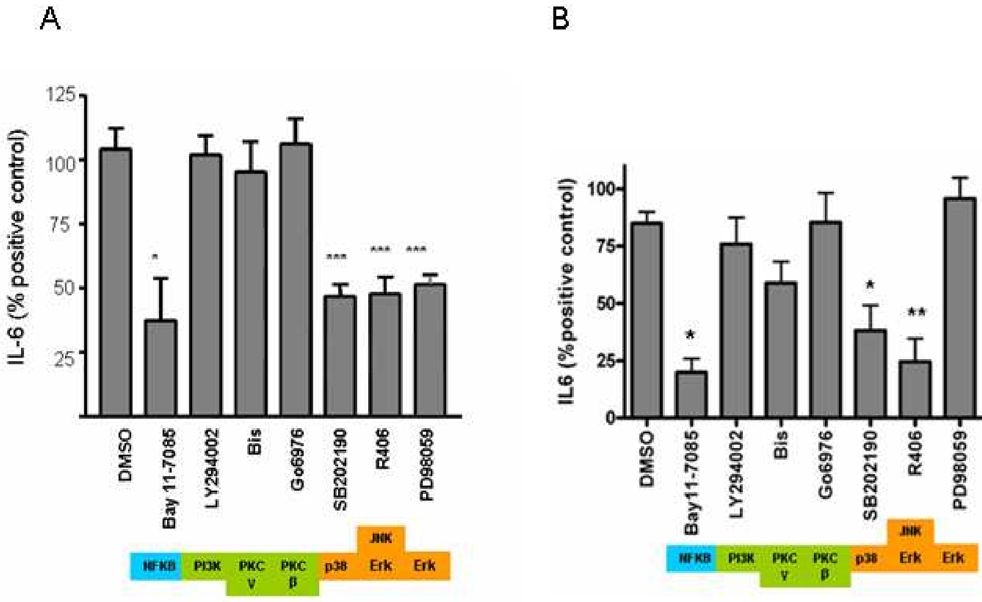


Fig. 6.5 Inhibition of IL-6 production in galectin-3 stimulated synovial and skin fibroblasts.

Fibroblasts from (A) synovium or (B) skin were treated with inhibitors for 1h prior to addition of medium containing 10 µg/ml galectin-3 or galectin-3 and DMSO and cells were cultured for 24h. IL-6 levels were measured by ELISA and expressed as percentage of production by positive (galectin-3-stimulated) control. Data are mean +SEM of 4-5 independent experiments and assayed in duplicate. *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared to control cells (DMSO); Bis = bisindolylmaleimide 1. Boxes indicate target kinases for the inhibitors.

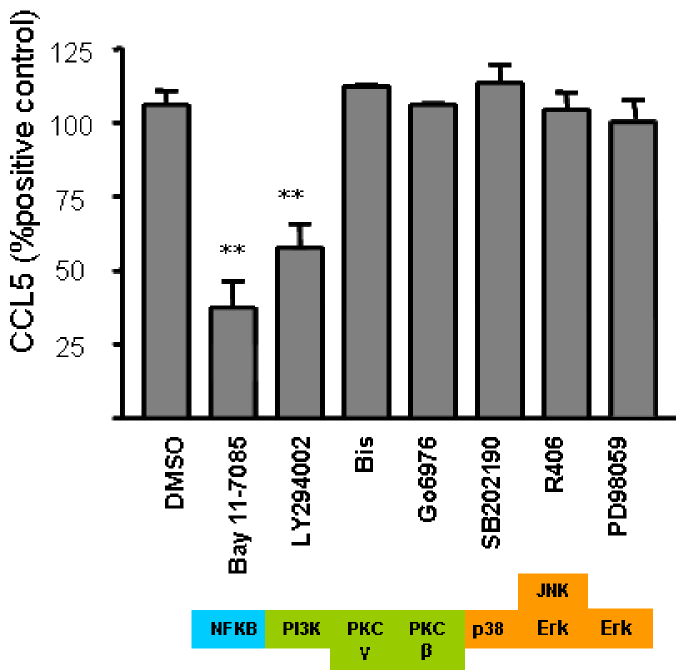


Fig. 6.6 Inhibition of CCL5 production in galectin-3 stimulated synovial fibroblasts.

Fibroblasts were treated with inhibitors for 1h prior to addition of medium containing 10 µg/ml galectin-3 or galectin-3 and DMSO as a control and cells were cultured for 24h. CCL5 levels were measured by ELISA and expressed as percentage of production by positive control (galectin-3-stimulated). Data are mean +SEM from 4 independent experiments assayed in duplicate. **$p<0.01$ compared to control cells (DMSO); Bis = bisindolylmaleimide 1. Boxes indicate target kinases for the inhibitors.

To confirm the activation of identified signalling pathways the phosphorylation of selected kinases was assessed by Western blotting using phosphospecific antibodies. Synovial and skin fibroblasts were treated with 10 μg/ml galectin-3 for 5 to 60 min, then cell lysates were collected and the expression of phosphorylated (active) and total protein kinase was examined. As presented in Fig. 6.7 increased phosphorylation of ERK kinase in synovial and skin fibroblasts was seen as early as 5 min. after addition of galectin-3 and lasted for at least 1h. Transient activation of p38 MAP kinase was observed in both types of fibroblasts after 15 min of galectin-3 treatment. JNK phosphorylation was also maximal at this time point then gradually decreased. A similar pattern was seen for Akt phosphorylation in skin fibroblasts. Probing for unphosphorylated proteins confirmed that gels were equally loaded.

Activation of NFκB transcription factor was measured after 1h of galectin-3 treatment. Stimulation with 10ng/ml TNFα was used as positive control. Synovial fibroblasts showed significant although variable activation of the p65 subunit (Fig. 6.8A). In skin fibroblasts the slight increase in active p65 (1.7 fold) in galectin-3-treated cells was not significant, possibly due to the low number of samples (Fig. 6.8B).

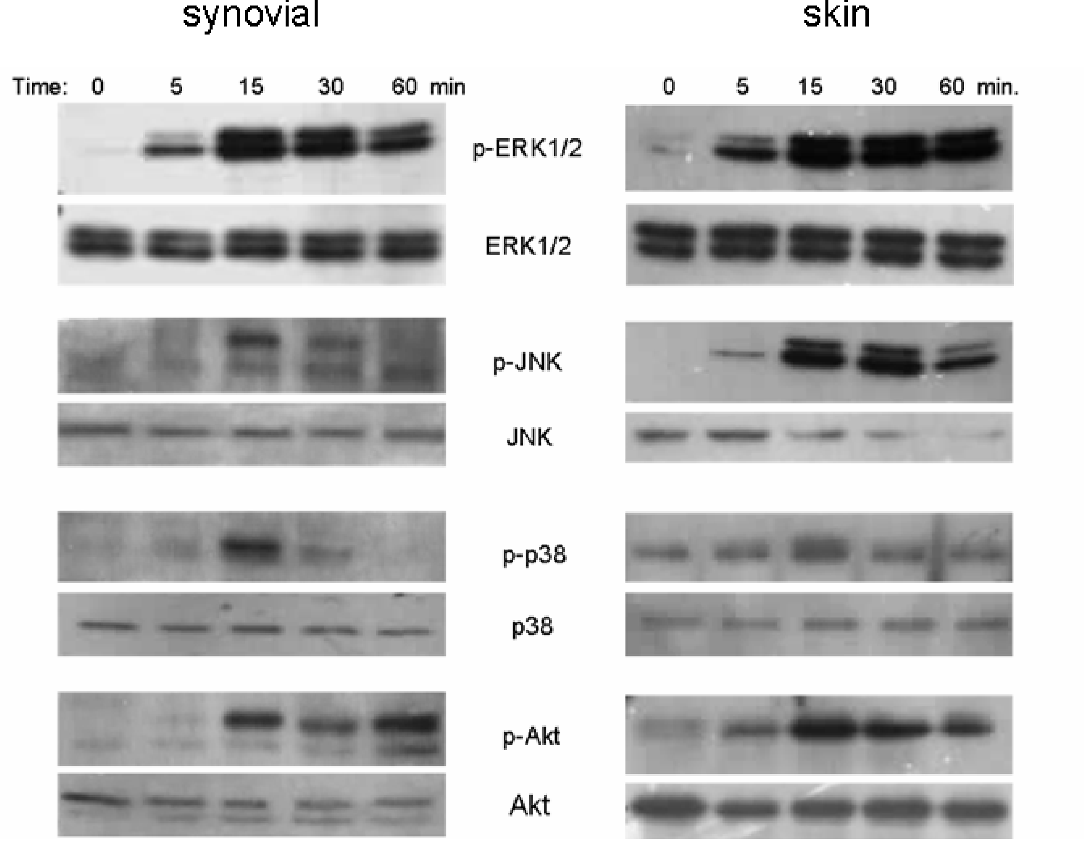


Fig. 6.7 Activation of protein kinases following galectin-3 treatment in synovial and skin fibroblasts.

Synovial and skin fibroblasts were treated with 10 μg/ml galectin-3 for 5-60 min. Cell lysates were prepared and analysed by Western blotting with antibodies against the total or phosphorylated (active) form of various protein kinases. Representative images from four separate experiments are shown for synovial (left panel) and skin (right panel) fibroblasts.

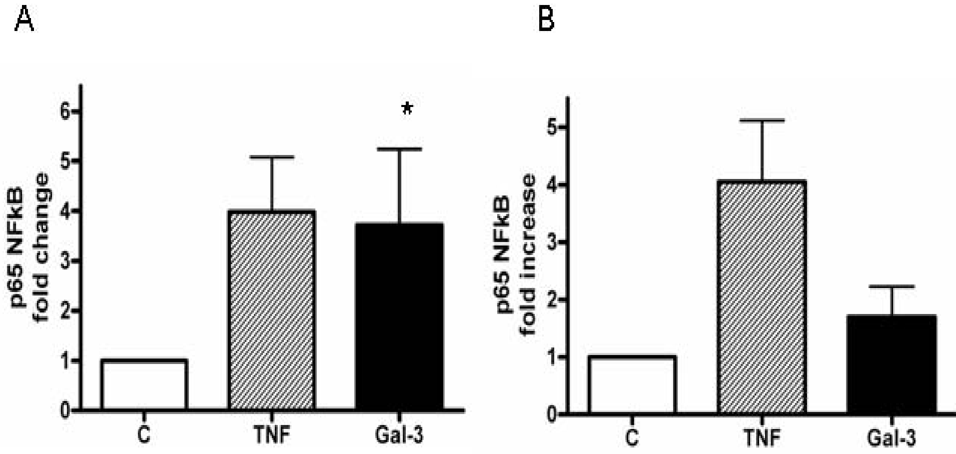


Fig. 6.8 Activation of NFκB transcription factor in synovial and skin fibroblasts. Fibroblasts were treated for 1h with 10 ng/ml TNFα or 10 μg/ml galectin-3. Nuclear extracts were prepared and the binding of activated NFκB p65 subunit to DNA oligonucleotides measured using the commercial TransAM assay kit. Data are expressed as fold change over untreated cells and are mean +SD from 3-4 experiments run in duplicate. * $p<0.05$ compared to control (C) non-treated cells.

6.5 DISCUSSION

Studies on the effects of in vitro treatment with galectin-3 showed increased production of IL-6 in synovial and skin fibroblasts from RA patients. Mononuclear cell-attracting chemokines (CCL5, CCL2) were selectively induced in fibroblasts from the synovium but not from the skin. These effects were not secondary events mediated by TNFα released from fibroblasts. It was also found the MAP kinase and NFκB signalling pathways were involved in IL-6 production, whereas CCL5 release was mediated by PI3K/Akt and NFκB pathways.

Galectin-3 was demonstrated to have a destructive potential in joint tissues (Janelle-Montcalm et al., 2007). It induced degradative enzymes from chondrocytes and inhibited osteoblast differentiation. Recent findings from our laboratory indicated another role for galectin-3 in the pathogenic role of rheumatoid synovium which is the induction of a variety of cytokines, chemokines, growth factors and proteases from synovial fibroblasts (Filer et al., 2009). Interestingly, no effect of galectins on fibroblast differentiation and surface molecule expression was observed (Fitton, 2007). Unlike TNF-α stimulation which induces various chemokines also in skin fibroblasts (Sticherling et al., 1995; Noso et al., 1996), galectin-3 enhanced their production only from synovial fibroblasts. CCL2 (MCP-1), CCL3 (MIP-1α) or CCL5 (RANTES) play a crucial role in the chemoattraction of leukocytes during the inflammatory response and have been implicated in rheumatoid arthritis (Rathanaswami et al., 1993). Their selective induction by galectin-3 present in the synovium may therefore contribute to the accumulation of an inflammatory infiltrate and initiation of the disease process at this site. Skin fibroblasts responded to

galectin-3 with production of cytokines (IL-6) but not chemokines. Only in one cell line was an increase in CCL5 noted and only at the highest doses of galectin. The differential response of skin fibroblasts may reflect the fact that they were derived from a non-inflamed tissue. On the other hand, the inflammatory process in RA synovium may have imprinted synovial fibroblasts causing their stable activation and ability to produce chemokines.

Although many binding partners for galectin-3 were described, a specific receptor for this lectin on fibroblasts has not been identified yet. It is possible that galectin-3 interacts with several molecules on the cell surface of fibroblasts and does not have a unique receptor. Therefore the approach taken in this study to determine the mechanism of cytokine and chemokine induction by galectin-3 was to look into signalling pathways potentially involved in IL-6 and CCL5 production. The hypothesis that differential production of these inflammatory cytokines and chemokines is driven by distinct signalling pathways was confirmed. The main transcription factor involved in regulation of IL-6 expression in synovial fibroblasts is NFκB (Georganas et al., 2000). However, upstream kinases leading to NFκB activation and IL-6 transcription appear to be dependent on a particular stimulus and receptor engaged. For instance, TNF-α or IL-1β signalling is mediated by p38 MAPK (Kunisch et al., 2007; Schett et al., 2000; Miyazawa et al., 1998), CCL2 stimulation enhances ERK1/2 phosphorylation (Nanki et al., 2001), whereas IL-6 induced by IL-17 activates PI3K/Akt pathway (Hwang et al., 2004).

Galectin-3 stimulation of synovial and skin fibroblasts activated each of the main MAP kinase families: p38MAPK, ERK and JNK as well as Akt phoshorylation.

NFκB transcription factor was also activated in galectin-3 treated synovial fibroblasts. An inhibitor of NF-κB signalling significantly reduced IL-6 secretion from both types of fibroblasts confirming that this transcription factor is crucial for IL-6 production in RA. Using protein kinase inhibitors it was also found that galectin-3-induced secretion of IL-6 in synovial fibroblasts required signalling by MAP kinases p38, JNK and ERK. Similar synergistic actions of p38 MAP kinase and NF-κB in induction of IL-6 was observed in cardiac myocytes (Craig et al., 2000). Apart from enhancing transcriptional activity p38 MAP kinase may also regulate IL-6 via stabilisation of its mRNA (Miyazawa et al., 1998).

Remarkably, in skin fibroblasts ERK1/2 kinases were also activated, but the inhibition of the direct upstream ERK activator MEK1 had no effect on IL-6 output. Indeed, other studies suggested involvement of different pathways in regulating ERK phosphorylation (Cerioni et al., 2003) and the MEK1 inhibitor PD98059 was ineffective in inhibiting ERK phosphorylation in rat-1 fibroblasts (Bapat et al., 2001). Induction of IL-6 may therefore show both receptor and tissue specific signalling pathway involvement.

Although the inhibitor of NFκB signaling (Bay 11-7085) reduced IL-6 production in skin fibroblasts similarly as it did in the synovial cells, the present data failed to confirm activation of this transcription factor. RA synovial fibroblasts display enhanced activation of this factor which was shown to contribute to their growth, resistance to apoptosis and transformed phenotype (Ospelt et al., 2004b; Miagkov et al., 1998; Li and Makarov, 2006). In skin-derived cells the threshold for NFκB

induction may be higher and galectin-3 may not provide a sufficient signal in this respect. Alternatively, other molecules from the complex intracellular signalling networks that were not considered in this study may be involved. The observation of weaker NFκB induction could also explain the lack of chemokine production in skin fibroblasts upon galectin-3 stimulation.

It was reported previously that CCL5 gene expression in fibroblasts requires NFκB (Genin et al., 2000) and JNK activation. CCL5 production induced by IL-1β or TNF-α involves also Syk, a spleen tyrosine kinase upstream of JNK (Yamada et al., 2001; Cha et al., 2006). Serine/threonine kinases from the PKC family (epsilon, zeta) have also been implicated in IL-1β/TNFα–induced chemokine production in synovial cells (Jordan et al., 1996) and were therefore considered here. We did not see any effect of PKC inhibitors (Bisindolylmaleimide 1, Go6976) on either IL-6 or CCL5 production.

In contrast to IL-6 production, CCL5 was affected only by PI3K and NFκB inhibitors. Therefore it can be concluded that in RA synovial fibroblasts the production of cytokines and chemokines is driven via distinct signaling pathways. A similar effect was observed previously for IL-18 driven production of angiogenic factors from these cells (Amin et al., 2007). In skin fibroblasts chemokines including CCL5 can be induced by stimuli such as TNFα, IL-1α/β or IFNγ (Sticherling et al., 1995; Fukuoka et al., 1998; Lee et al., 2000). The fact that galectin-3 promoted mononuclear-attracting chemokine expression only in synovial fibroblasts may contribute to its inflammatory role at this specific site.

7.0 GENERAL DISCUSSION

Fibroblast derived neutrophil survival factors

The first part of this thesis was an attempt to identify neutrophil survival factors secreted by synovial fibroblasts in rheumatoid arthritis. It stemmed from previous observations of enhanced neutrophil survival elicited by TNFα and IL-17-stimulated fibroblasts (Filer et al., 2006a). Stromally-induced leukocyte survival appears to be important in sustaining inflammatory processes in the tissues. In RA synovium it was shown that such a mechanism applies to both plasma cells and T cells. Synovial fibroblasts provide functional niches for B cells where their survival and differentiation into plasma cells is provided mainly by direct cell contact (Dechanet et al., 1995a). T cells also are believed to survive and accumulate in the joint due to cell contact with fibroblasts and the presence of soluble factors secreted by them (Pilling et al., 1999). Neutrophils in the RA joint are found mostly in synovial fluid and at the interface of pannus and cartilage (Edwards and Hallett, 1997) and therefore it was less clear whether they received survival signals from stromal cells. However, work from our group showed that fibroblasts could provide survival factors able to inappropriately retain these cells in the joint (Filer et al., 2006a). The identity of the survival factors was however not known and was one of the subjects of this thesis.

RA synovial fibroblasts secreted soluble molecules prolonging neutrophil life span. As shown previously and confirmed herein, GM-CSF accounted for much of this effect (Filer, 2006, Parsonage et al., 2008). In search for other mediators that provide survival signals for neutrophils, it was shown that the unknown factor is heat-labile,

therefore most likely a protein, and acts by activating PI-3K and NFκB signalling pathways in neutrophils (Filer, 2006). However, such other known survival factors as G-CSF, CCL2 were excluded. To further pursue the apoptosis delaying agent this study examined the role of IFNβ, IL-6, TNFα and galectin-3, but none of them appeared to contribute to the survival effect produced by TNF and IL-17 stimulated fibroblasts. Despite eliminating a number of candidates, the issue of fibroblast-produced neutrophil survival factor has not been successfully resolved. Another way forward to identify this molecule could be fractionation of the supernatant and mass spectrometry to decide its molecular weight or microarray analysis of unstimulated and stimulated fibroblasts to see which genes of candidate survival factors are differentially expressed.

Differential fibroblast phenotypes

The role of fibroblasts as tissue-resident sentinel cells that integrate signals from the environment and translate into immune function by modulating leukocyte behaviour is gaining more interest. The research from our group focuses also on defining unique phenotypes characteristic for fibroblasts originating from various anatomical sites. It was shown that molecular profiles of these stromal cells indeed differ (Parsonage et al., 2003) and may constitute a basis for development of chronic inflammation at specific restricted sites such as the joint in rheumatoid arthritis. The observation of similarities in cytokine secretory patterns and responses to various stimuli among fibroblasts from rheumatoid, osteoarthritic and normal synovium suggests that site of origin may even be more important that disease specificity in defining fibroblast phenotype (Scaife et al., 2004).

This thesis sought to elucidate whether galectins as immunomodulatory molecules constitute a part of this site-specific expression profile in fibroblasts and if their expression can be changed by external stimuli. Galectins 1, 3 and 8 were found to be highly expressed in fibroblasts regardless of their origin. Site-specific differences in expression patterns were observed for galectin-9 and -12. Up to now expression of this latter galectin had not been reported in RA fibroblasts and its significance requires further investigation.

Role of galectin-9 in rheumatoid joint

Galectin-9 was recognized initially as an eosinophil chemoattractant and T cell survival regulator. Being a ligand for TIM-3 receptor on Th1 cells it can induce T cell apoptosis which has substantial implications in development of autoimmune disease, graft survival or anti-tumour activity (Wang et al., 2007; Seki et al., 2007). During the course of this thesis a publication by Seki et al. showed the presence of galectin-9 in RA synovium and its proapoptotic role for synovial fibroblasts and potential beneficial effect of such treatment in arthritis (Seki et al., 2007). The present findings of higher expression of galectin-9 in synovial fibroblasts compared to those from skin, does not support their putative pro-apoptotic function in these fibroblasts. However, the authors of the above mentioned study used a mutated molecule derived from galectin-9 which may provide an explanation for this discrepancy.

This thesis focused on intracellular galectin-9 and provides a different view of the role of this protein in synovial fibroblast biology. It was shown that galectin-9 silencing resulted in increased rates of apoptosis in fibroblasts implicating a

protective role of the intracellular protein. The mechanism of this protection would be an interesting subject to investigate further. Involvement of galectin-9 in specific apoptosis signalling pathways should be examined, for example its association with regulatory proteins such as Bcl-2, Bcl-xl or caspases. Overexpression of galectin-9 in fibroblasts and the effect on resistance to apoptotic stimuli would confirm the role of this molecule.

The data from in situ examination of galectin-9 expression in synovial tissue demonstrated its nuclear localisation. So far this galectin has not been reported in fibroblasts in the nuclear compartment and its particular role there is not known. However, another recent publication noted nuclear translocation of galectin-9 in monocytic cells challenged with LPS and its physical association with NF-IL6 (C/EBPβ) transcription factor (Matsuura et al., 2009) suggesting involvement in the transcription machinery. Indeed, the presence of galectin-9 in those cells induced pro-inflammatory cytokine production (IL-1α, IL-1β) (Matsuura et al, 2009). Here we also report an upregulation of galectin-9 in response to LPS, although without an effect on the few inflammatory molecules tested (IL-6, CCL5, IL-8, MMP3).

The abundant expression of galectin-9 at sites of pannus invasion into the cartilage and bone together with its anti-apoptotic activity indicates a potential role in the aggressive behaviour of RA synovial fibroblasts. Since the exogenous galectin-9 could induce fibroblast apoptosis (Seki et al., 2007), this finding underlines that the localization of galectin-9 and whether it is secreted in a soluble form or not, may be of crucial importance for its in vivo activities in the inflamed joint. Further investigation on this subject such as measuring the levels of galectin-9 and testing the effects of a native protein would be necessary to clarify this issue.

Role of galectin-3 in rheumatoid joint

Another galectin with a role in rheumatoid arthritis and present in the synovium at much higher levels is galectin-3. It was revealed that apart from fibroblasts other cells such as macrophages contribute to its production and increased levels in RA. Galectin-3 was shown to be a critical regulator of chronic inflammation in liver or renal fibrosis (Henderson et al., 2006; Henderson et al., 2008). Here it is proposed that galectin-3 may contribute to the pathogenesis of rheumatoid arthritis by directly stimulating fibroblasts to secrete leukocyte recruiting and survival factors (Filer et al., 2009). We also provide the evidence for site-specific responses of fibroblasts depending on their origin (synovium or skin). The production of mononuclear attracting chemokines by fibroblasts from the synovium but not from skin is a unique property of galectin-3 stimulation. Although the receptor(s) on both cell types are not yet known it was found that the secretion of inflammatory mediators is based on the activation of distinct pathways, PI3K/Akt in case of chemokines and MAPKs in case of cytokines. These differences in intracellular signaling pathways and various outcomes of their activation in synovial and skin fibroblasts adds to the concept of spatially defined imprinted phenotypes of fibroblasts.

Implications for treatment of RA

Although the role of galectins in rheumatoid arthritis is only beginning to emerge they might constitute another target for therapy. Current therapies for RA aim to slow down progression of the disease and treat the symptoms but do not represent a complete cure. They include traditional broad range anti-inflammatory agents such as

corticosteroids and cytostatic drugs and recently developed biological agents (Table 7.1). The most successful biologics utilized for the treatment of rheumatoid arthritis antagonize pro-inflammatory cytokines primarily TNFα. IL-1 and IL-6 receptor antagonists as well as molecules targeting immune cells (T cells, B cells) were also developed and proven beneficial in controlling the disease.

Table 7.1. Current primary medications used to treat RA.

Analgesics/NSAIDs	**DMARDs**	**Biologics**	
Acetaminophen	Azathioprine	Etanercept	fusion protein TNFR:IgG
Tramadol	D-penicillamine	Infliximab	chimeric anti-TNFα antibody
Cox-2 inhibitors	Hydroxychloroquine	Adalimumab	human anti-TNFα antibody
Capsaicin	Sulfasalazine	Anakinra	recombinant IL-1Ra
Narcotics	Methotrexate	Rituximab	anti-CD20 antibody
Ibuprofen	Leflunomide	Abatacept	fusion protein CTLA-4:IgG Fc
	Gold salts	Tocilizumab	anti- IL-6R antibody

NSAIDs - nonsteroidal anti-inflammatory drugs
DMARDs –disease-modyfying antirheumatic drugs

However, even with these treatments still only about 60-70% of RA patients show clinical improvement. The reason for this might be that most of the current therapies focus on targeting the adaptive immune system without tackling the innate immunity such as neutrophils and stromal cells. Fibroblasts are now recognized as important players in inflammatory processes not only in the joint. Their huge capacity to attract and activate cells of the adaptive system as well as responsiveness to stimuli provided by incoming immune cells make them indispensable to sustain inflammation in the tissues. As shown in this thesis fibroblasts may be crucially responsible for maintaining neutrophil infiltrate in the joint. Targeting them to remove the source of factors causing inappropriate neutrophil survival would present additional option for treatment of RA.

In addition, synovial fibroblasts are an important source of inflammatory galectin-3 which also acts in an autocrine manner causing their further activation and triggering cytokine and chemokine production. It can be concluded that interfering with this pathway could restrict synovial inflammation and aid to improve available treatment strategies.

Finally, another galectin produced by fibroblasts, galectin-9 may contribute to their resistance to apoptosis. This should also be considered before attempting to utilize galectin-9 as a therapeutical agent.

Reference List

Abdollahi-Roodsaz,S., Joosten,L.A., Roelofs,M.F., Radstake,T.R., Matera,G., Popa,C., van der Meer,J.W., Netea,M.G., and van den Berg,W.B. (2007). Inhibition of Toll-like receptor 4 breaks the inflammatory loop in autoimmune destructive arthritis. Arthritis Rheum. *56*, 2957-2967.

Abedin,M.J., Kashio,Y., Seki,M., Nakamura,K., and Hirashima,M. (2003). Potential roles of galectins in myeloid differentiation into three different lineages. J Leukoc Biol. *73*, 650-656.

Abreu,J.R., Grabiec,A.M., Krausz,S., Spijker,R., Burakowski,T., Maslinski,W., Eldering,E., Tak,P.P., and Reedquist,K.A. (2009). The Presumed Hyporesponsive Behavior of Rheumatoid Arthritis T Lymphocytes Can Be Attributed to Spontaneous Ex Vivo Apoptosis rather than Defects in T Cell Receptor Signaling. J. Immunol.

Adams,L., Scott,G.K., and Weinberg,C.S. (1996). Biphasic modulation of cell growth by recombinant human galectin-1. Biochim. Biophys. Acta. *1312*, 137-144.

Ahmad,N., Gabius,H.J., Andre,S., Kaltner,H., Sabesan,S., Roy,R., Liu,B., Macaluso,F., and Brewer,C.F. (2004). Galectin-3 precipitates as a pentamer with synthetic multivalent carbohydrates and forms heterogeneous cross-linked complexes. J Biol. Chem. *19;279*, 10841-10847.

Ahmed,H., Banerjee,P.P., and Vasta,G.R. (2007). Differential expression of galectins in normal, benign and malignant prostate epithelial cells: Silencing of galectin-3 expression in prostate cancer by its promoter methylation. Biochemical and Biophysical Research Communications *358*, 241-246.

Akahani,S., Nangia-Makker,P., Inohara,H., Kim,H.R., and Raz,A. (1997). Galectin-3: a novel antiapoptotic molecule with a functional BH1 (NWGR) domain of Bcl-2 family. Cancer Res. *57*, 5272-5276.

Akira,S., Takeda,K., and Kaisho,T. (2001). Toll-like receptors: critical proteins linking innate and acquired immunity. Nat. Immunol. *2*, 675-680.

Almkvist,J. and Karlsson,A. (2004). Galectins as inflammatory mediators. Glycoconj. J. *19*, 575-581.

Almkvist,J., Dahlgren,C., Leffler,H., and Karlsson,A. (2002). Activation of the Neutrophil Nicotinamide Adenine Dinucleotide Phosphate Oxidase by Galectin-1. J Immunol *168*, 4034-4041.

Alsalameh,S., Amin,R.J., Kunisch,E., Jasin,H.E., and Kinne,R.W. (2003). Preferential induction of prodestructive matrix metalloproteinase-1 and proinflammatory interleukin 6 and prostaglandin E2 in rheumatoid arthritis synovial fibroblasts via tumor necrosis factor receptor-55. J. Rheumatol. *30*, 1680-1690.

Alsayed,Y., Uddin,S., Ahmad,S., Majchrzak,B., Druker,B.J., Fish,E.N., and Platanias,L.C. (2000). IFN-{gamma} Activates the C3G/Rap1 Signaling Pathway. J Immunol *164*, 1800-1806.

Alvaro-Gracia,J.M., Zvaifler,N.J., and Firestein,G.S. (1990). Cytokines in chronic inflammatory arthritis. V. Mutual antagonism between interferon-gamma and tumor necrosis factor-alpha on HLA-DR expression, proliferation, collagenase production, and granulocyte macrophage colony-stimulating factor production by rheumatoid arthritis synoviocytes. J Clin. Invest. *86*, 1790-1798.

Amin,M.A., Mansfield,P.J., Pakozdi,A., Campbell,P.L., Ahmed,S., Martinez,R.J., and Koch,A.E. (2007). Interleukin-18 induces angiogenic factors in rheumatoid arthritis synovial tissue fibroblasts via distinct signaling pathways. Arthritis Rheum. *56*, 1787-1797.

Andre,S., Kojima,S., Yamazaki,N., Fink,C., Kaltner,H., Kayser,K., and Gabius,H.J. (1999). Galectins-1 and -3 and their ligands in tumor biology. Non-uniform properties in cell-surface presentation and modulation of adhesion to matrix glycoproteins for various tumor cell lines, in biodistribution of free and liposome-bound galectins and in their expression by breast and colorectal carcinomas with/without metastatic propensity. J Cancer Res Clin Oncol. *125*, 461-474.

Arend,W.P. and Gabay,C. (2006). Cytokine networks. In Rheumatoid arthritis, Oxford University Press).

Arnett,F.C., Edworthy,S.M., Bloch,D.A., McShane,D.J., Fries,J.F., Cooper,N.S., Healey,L.A., Kaplan,S.R., Liang,M.H., Luthra,H.S., and . (1988). The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum. *31*, 315-324.

Asahara,H., Fujisawa,K., Kobata,T., Hasunuma,T., Maeda,T., Asanuma,M., Ogawa,N., Inoue,H., Sumida,T., and Nishioka,K. (1997). Direct evidence of high DNA binding activity of transcription factor AP-1 in rheumatoid arthritis synovium. Arthritis Rheum. *40*, 912-918.

Asakura,H., Kashio,Y., Nakamura,K., Seki,M., Dai,S., Shirato,Y., Abedin,M.J., Yoshida,N., Nishi,N., Imaizumi,T., Saita,N., Toyama,Y., Takashima,H., Nakamura,T., Ohkawa,M., and Hirashima,M. (2002). Selective Eosinophil Adhesion to Fibroblast Via IFN-{gamma}-Induced Galectin-9. J Immunol *169*, 5912-5918.

Bapat,S., Verkleij,A., and Post,J.A. (2001). Peroxynitrite activates mitogen-activated protein kinase (MAPK) via a MEK-independent pathway: a role for protein kinase C. FEBS Lett. *499*, 21-26.

Barboni,E.A., Bawumia,S., and Hughes,R.C. (1999). Kinetic measurements of binding of galectin 3 to a laminin substratum. Glycoconj. J. *16*, 365-373.

Barondes,S.H., Cooper,D.N., Gitt,M.A., and Leffler,H. (1994). Galectins. Structure and function of a large family of animal lectins. J. Biol. Chem. *269*, 20807-20810.

Barrionuevo,P., Beigier-Bompadre,M., Ilarregui,J.M., Toscano,M.A., Bianco,G.A., Isturiz,M.A., and Rabinovich,G.A. (2007). A novel function for galectin-1 at the crossroad of innate and adaptive immunity: galectin-1 regulates monocyte/macrophage physiology through a nonapoptotic ERK-dependent pathway. J. Immunol. *178*, 436-445.

Baum,L.G., Blackall,D.P., Arias-Magallano,S., Nanigian,D., Uh,S.Y., Browne,J.M., Hoffmann,D., Emmanouilides,C.E., Territo,M.C., and Baldwin,G.C. (2003). Amelioration of graft versus host disease by galectin-1. Clin. Immunol. *109*, 295-307.

Benbow,U. and Brinckerhoff,C.E. (1997). The AP-1 site and MMP gene regulation: what is all the fuss about? Matrix Biol. *15*, 519-526.

Benvenuto,G., Carpentieri,M.L., Salvatore,P., Cindolo,L., Bruni,C.B., and Chiariotti,L. (1996). Cell-specific transcriptional regulation and reactivation of galectin-1 gene expression are controlled by DNA methylation of the promoter region [published erratum appears in Mol Cell Biol 1996 Aug;16(8):4590]. Mol. Cell. Biol. *16*, 2736-2743.

Berberat,P.O., Friess,H., Wang,L., Zhu,Z., Bley,T., Frigeri,L., Zimmermann,A., and Buchler,M.W. (2001). Comparative analysis of galectins in primary tumors and tumor metastasis in human pancreatic cancer. J Histochem. Cytochem. *49*, 539-549.

Bidon,N., Brichory,F., Bourguet,P., Le Pennec,J.P., and Dazord,L. (2001). Galectin-8: a complex sub-family of galectins (Review). Int. J Mol. Med. *8*, 245-250.

Blaser,C., Kaufmann,M., Muller,C., Zimmermann,C., Wells,V., Mallucci,L., and Pircher,H. (1998). Beta-galactoside-binding protein secreted by activated T cells inhibits antigen-induced proliferation of T cells. Eur. J Immunol. *28*, 2311-2319.

Blom,A.B., Radstake,T.R., Holthuysen,A.E., Sloetjes,A.W., Pesman,G.J., Sweep,F.G., van de Loo,F.A., Joosten,L.A., Barrera,P., van Lent,P.L., and van den Berg,W.B. (2003). Increased expression of Fcgamma receptors II and III on macrophages of rheumatoid arthritis patients results in higher production of tumor necrosis factor alpha and matrix metalloproteinase. Arthritis Rheum. *48*, 1002-1014.

Borrello,M.A. and Phipps,R.P. (1996). Differential Thy-1 expression by splenic fibroblasts defines functionally distinct subsets. Cell Immunol. *173*, 198-206.

Bradfield,P.F., Amft,N., Vernon-Wilson,E., Exley,A.E., Parsonage,G., Rainger,G.E., Nash,G.B., Thomas,A.M., Simmons,D.L., Salmon,M., and Buckley,C.D. (2003). Rheumatoid fibroblast-like synoviocytes overexpress the chemokine stromal cell-derived factor 1 (CXCL12), which supports distinct patterns and rates of CD4+ and CD8+ T cell migration within synovial tissue. Arthritis Rheum. *48*, 2472-2482.

Brandt,B., Buchse,T., bou-Eladab,E.F., Tiedge,M., Krause,E., Jeschke,U., and Walzel,H. (2008). Galectin-1 induced activation of the apoptotic death-receptor pathway in human Jurkat T lymphocytes. Histochem. Cell Biol. *129*, 599-609.

Brennan,F. and Foey,A. (2002). Cytokine regulation in RA synovial tissue: role of T cell/macrophage contact-dependent interactions. Arthritis Res. *4 Suppl 3:S177-82. Epub;%2002 May 9.*, S177-S182.

Brennan,F.M., Maini,R.N., and Feldmann,M. (1998). Role of pro-inflammatory cytokines in rheumatoid arthritis. Springer Semin. Immunopathol. *20*, 133-147.

Brentano,F., Schorr,O., Gay,R.E., Gay,S., and Kyburz,D. (2005). RNA released from necrotic synovial fluid cells activates rheumatoid arthritis synovial fibroblasts via Toll-like receptor 3. Arthritis Rheum. *52*, 2656-2665.

Brouty-Boye,D., Pottin-Clemenceau,C., Doucet,C., Jasmin,C., and Azzarone,B. (2000). Chemokines and CD40 expression in human fibroblasts. Eur. J. Immunol. *30*, 914-919.

Bucala,R., Ritchlin,C., Winchester,R., and Cerami,A. (1991). Constitutive production of inflammatory and mitogenic cytokines by rheumatoid synovial fibroblasts. J Exp. Med. *173*, 569-574.

Buch,M.H., Vital,E.M., and Emery,P. (2008). Abatacept in the treatment of rheumatoid arthritis. Arthritis Res. Ther. *10 Suppl 1:S5. Epub;%2008 Oct 15.*, S5.

Buckley,C.D. (2003a). Why does chronic inflammatory joint disease persist? Clin. Med. *3*, 361-366.

Buckley,C.D. (2003b). Michael Mason prize essay 2003. Why do leucocytes accumulate within chronically inflamed joints? Rheumatology. (Oxford). *42*, 1433-1444.

Buckley,C.D., Filer,A., Haworth,O., Parsonage,G., and Salmon,M. (2004a). Defining a role for fibroblasts in the persistence of chronic inflammatory joint disease. Ann. Rheum. Dis. *63 Suppl 2:ii92-ii95.*, ii92-ii95.

Buckley,C.D., Pilling,D., Lord,J.M., Akbar,A.N., Scheel-Toellner,D., and Salmon,M. (2001). Fibroblasts regulate the switch from acute resolving to chronic persistent inflammation. Trends Immunol. *22*, 199-204.

Burger,J.A., Zvaifler,N.J., Tsukada,N., Firestein,G.S., and Kipps,T.J. (2001). Fibroblast-like synoviocytes support B-cell pseudoemperipolesis via a stromal cell-derived factor-1- and CD106 (VCAM-1)-dependent mechanism. J. Clin. Invest. *107*, 305-315.

Burman,A., Haworth,O., Bradfield,P., Parsonage,G., Filer,A., Thomas,A.M., Amft,N., Salmon,M., and Buckley,C.D. (2005). The role of leukocyte-stromal interactions in chronic inflammatory joint disease. Joint Bone Spine. *72*, 10-16.

Calvo-Alen,J. and Alarcon,G.S. (2006). Epidemiology and determinants of suscebility. In Rheumatoid arthrits, G.S.Firestein, G.S.Panayi, and F.A.Wollheim, eds. Oxford Univesity Press), pp. 15-29.

Camby,I., Le Mercier,M., Lefranc,F., and Kiss,R. (2006). Galectin-1: a small protein with major functions. Glycobiology. *16*, 137R-157R.

Cassatella,M.A., Gasperini,S., and Russo,M.P. (1997). Cytokine expression and release by neutrophils. Ann N. Y. Acad. Sci. *832:233-42.*, 233-242.

Cerioni,L., Palomba,L., and Cantoni,O. (2003). The Raf/MEK inhibitor PD98059 enhances ERK1/2 phosphorylation mediated by peroxynitrite via enforced mitochondrial formation of reactive oxygen species. FEBS Lett. *547*, 92-96.

Cha,H.S., Boyle,D.L., Inoue,T., Schoot,R., Tak,P.P., Pine,P., and Firestein,G.S. (2006). A novel spleen tyrosine kinase inhibitor blocks c-Jun N-terminal kinase-mediated gene expression in synoviocytes. J Pharmacol. Exp. Ther. *317*, 571-578.

Chabaud,M., Durand,J.M., Buchs,N., Fossiez,F., Page,G., Frappart,L., and Miossec,P. (1999). Human interleukin-17: A T cell-derived proinflammatory cytokine produced by the rheumatoid synovium. Arthritis Rheum. *42*, 963-970.

Chabot,S., Kashio,Y., Seki,M., Shirato,Y., Nakamura,K., Nishi,N., Nakamura,T., Matsumoto,R., and Hirashima,M. (2002). Regulation of galectin-9 expression and release in Jurkat T cell line cells. Glycobiology *12*, 111-118.

Chang,H.Y., Chi,J.T., Dudoit,S., Bondre,C., van de,R.M., Botstein,D., and Brown,P.O. (2002). Diversity, topographic differentiation, and positional memory in human fibroblasts. Proc. Natl. Acad. Sci. U. S. A. *99*, 12877-12882.

Chen,H.Y., Sharma,B.B., Yu,L., Zuberi,R., Weng,I.C., Kawakami,Y., Kawakami,T., Hsu,D.K., and Liu,F.T. (2006). Role of galectin-3 in mast cell functions: galectin-3-deficient mast cells exhibit impaired mediator release and defective JNK expression. J. Immunol. *177*, 4991-4997.

Cheon,H., Yu,S.J., Yoo,D.H., Chae,I.J., Song,G.G., and Sohn,J. (2002). Increased expression of pro-inflammatory cytokines and metalloproteinase-1 by TGF-beta1 in synovial fibroblasts from rheumatoid arthritis and normal individuals. Clin. Exp. Immunol. *127*, 547-552.

Chiariotti,L., Salvatore,P., Frunzio,R., and Bruni,C.B. (2004). Galectin genes: regulation of expression. Glycoconj. J. *19*, 441-449.

Chomarat,P., Rissoan,M.C., Pin,J.J., Banchereau,J., and Miossec,P. (1995). Contribution of IL-1, CD14, and CD13 in the increased IL-6 production induced by in vitro monocyte-synoviocyte interactions. J. Immunol. *155*, 3645-3652.

Chung,C.D., Patel,V.P., Moran,M., Lewis,L.A., and Miceli,M.C. (2000). Galectin-1 Induces Partial TCR {zeta}-Chain Phosphorylation and Antagonizes Processive TCR Signal Transduction. J Immunol *165*, 3722-3729.

Colnot,C., Ripoche,M.A., Milon,G., Montagutelli,X., Crocker,P.R., and Poirier,F. (1998). Maintenance of granulocyte numbers during acute peritonitis is defective in galectin-3-null mutant mice. Immunology. *94*, 290-296.

Cooper,D.N. and Barondes,S.H. (1999). God must love galectins; he made so many of them. Glycobiology. *9*, 979-984.

Coxon,A., Cullere,X., Knight,S., Sethi,S., Wakelin,M.W., Stavrakis,G., Luscinskas,F.W., and Mayadas,T.N. (2001). Fc gamma RIII mediates neutrophil recruitment to immune complexes. a mechanism for neutrophil accumulation in immune-mediated inflammation. Immunity. *14*, 693-704.

Craig,R., Larkin,A., Mingo,A.M., Thuerauf,D.J., Andrews,C., McDonough,P.M., and Glembotski,C.C. (2000). p38 MAPK and NF-kappa B Collaborate to Induce Interleukin-6 Gene Expression and Release. EVIDENCE FOR A CYTOPROTECTIVE AUTOCRINE SIGNALING PATHWAY IN A CARDIAC MYOCYTE MODEL SYSTEM. J. Biol. Chem. *275*, 23814-23824.

Cross,A., Barnes,T., Bucknall,R.C., Edwards,S.W., and Moots,R.J. (2006). Neutrophil apoptosis in rheumatoid arthritis is regulated by local oxygen tensions within joints. J Leukoc Biol *80*, 521-528.

Cutolo,M. and Lahita,R.G. (2005). Estrogens and Arthritis. Rheumatic Disease Clinics of North America *31*, 19-27.

Daffern,P.J., Jagels,M.A., and Hugli,T.E. (1999). Multiple epithelial cell-derived factors enhance neutrophil survival. Regulation by glucocorticoids and tumor necrosis factor-alpha. Am. J. Respir. Cell Mol. Biol. *21*, 259-267.

Dagher,S.F., Wang,J.L., and Patterson,R.J. (1995). Identification of galectin-3 as a factor in pre-mRNA splicing. Proc. Natl. Acad. Sci. U. S. A. *92*, 1213-1217.

Dai,S.Y., Nakagawa,R., Itoh,A., Murakami,H., Kashio,Y., Abe,H., Katoh,S., Kontani,K., Kihara,M., Zhang,S.L., Hata,T., Nakamura,T., Yamauchi,A., and Hirashima,M. (2005). Galectin-9 Induces Maturation of Human Monocyte-Derived Dendritic Cells. J Immunol *175*, 2974-2981.

Danks,L., Sabokbar,A., Gundle,R., and Athanasou,N.A. (2002). Synovial macrophage-osteoclast differentiation in inflammatory arthritis. Ann Rheum Dis *61*, 916-921.

de Jong H., Berlo,S.E., Hombrink,P., Otten,H.G., van,E.W., Lafeber,F.P., Heurkens,A.H., Bijlsma,J.W., Glant,T.T., and Prakken,B. (2009). Cartilage proteoglycan aggrecan epitopes induce proinflammatory autoreactive T cell responses in rheumatoid arthritis and osteoarthritis. Ann. Rheum. Dis.

de Melo,F.H.M., Butera,D., Medeiros,R.S., Andrade,L.N., Nonogaki,S., Soares,F.A., Alvarez,R.A., Moura da Silva,A.M., and Chammas,R. (2007). Biological Applications of a Chimeric Probe for the Assessment of Galectin-3 Ligands. J. Histochem. Cytochem. *55*, 1015-1026.

Dechanet,J., Merville,P., Durand,I., Banchereau,J., and Miossec,P. (1995a). The ability of synoviocytes to support terminal differentiation of activated B cells may

explain plasma cell accumulation in rheumatoid synovium. J Clin. Invest. *95*, 456-463.

Dechanet,J., Rissoan,M.C., Banchereau,J., and Miossec,P. (1995b). Interleukin 4, but not interleukin 10, regulates the production of inflammation mediators by rheumatoid synoviocytes. Cytokine. *7*, 176-183.

Demers,M., Biron-Pain,K., Hebert,J., Lamarre,A., Magnaldo,T., and St-Pierre,Y. (2007). Galectin-7 in lymphoma: elevated expression in human lymphoid malignancies and decreased lymphoma dissemination by antisense strategies in experimental model. Cancer Res. *67*, 2824-2829.

Demetriou,M., Granovsky,M., Quaggin,S., and Dennis,J.W. (2001). Negative regulation of T-cell activation and autoimmunity by Mgat5 N-glycosylation. Nature. *409*, 733-739.

Dias-Baruffi,M., Zhu,H., Cho,M., Karmakar,S., McEver,R.P., and Cummings,R.D. (2003). Dimeric galectin-1 induces surface exposure of phosphatidylserine and phagocytic recognition of leukocytes without inducing apoptosis. J. Biol. Chem. *278*, 41282-41293.

Dube,D.H. and Bertozzi,C.R. (2005). GLYCANS IN CANCER AND INFLAMMATION - POTENTIAL FOR THERAPEUTICS AND DIAGNOSTICS. Nat Rev Drug Discov *4*, 477-488.

Dumic,J., Dabelic,S., and Flogel,M. (2006). Galectin-3: an open-ended story. Biochim. Biophys. Acta. *1760*, 616-635.

Dunican,A.L., Leuenroth,S.J., Grutkoski,P., Ayala,A., and Simms,H.H. (2000). TNFalpha-induced suppression of PMN apoptosis is mediated through interleukin-8 production. Shock. *14*, 284-288.

Dyer,K.D. and Rosenberg,H.F. (1996). Eosinophil Charcot-Leyden crystal protein binds to beta-galactoside sugars. Life Sci. *58*, 2073-2082.

Dyer,K.D. and Rosenberg,H.F. (2001). Transcriptional regulation of galectin-10 (eosinophil Charcot-Leyden crystal protein): a GC box (-44 to -50) controls butyric acid induction of gene expression. Life Sci. *69*, 201-212.

Edwards,J.C., Szczepanski,L., Szechinski,J., Filipowicz-Sosnowska,A., Emery,P., Close,D.R., Stevens,R.M., and Shaw,T. (2004). Efficacy of B-cell-targeted therapy with rituximab in patients with rheumatoid arthritis. N. Engl. J. Med. *350*, 2572-2581.

Edwards,S.W. and Hallett,M.B. (1997). Seeing the wood for the trees: the forgotten role of neutrophils in rheumatoid arthritis. Immunol Today. *18*, 320-324.

Edwards,S.W., Watson,F., Gasmi,L., Moulding,D.A., and Quayle,J.A. (1997). Activation of human neutrophils by soluble immune complexes: role of Fc gamma

RII and Fc gamma RIIIb in stimulation of the respiratory burst and elevation of intracellular Ca2+. Ann N. Y. Acad. Sci. *832:341-57.*, 341-357.

Elad-Sfadia,G., Haklai,R., Balan,E., and Kloog,Y. (2004). Galectin-3 Augments K-Ras Activation and Triggers a Ras Signal That Attenuates ERK but Not Phosphoinositide 3-Kinase Activity. J. Biol. Chem. *279*, 34922-34930.

Elias,J.A., Jimenez,S.A., and Freundlich,B. (1987). Recombinant gamma, alpha, and beta interferon regulation of human lung fibroblast proliferation. Am. Rev. Respir. Dis. *135*, 62-65.

Ellerhorst,J., Nguyen,T., Cooper,D.N., Lotan,D., and Lotan,R. (1999). Differential expression of endogenous galectin-1 and galectin-3 in human prostate cancer cell lines and effects of overexpressing galectin-1 on cell phenotype. Int. J Oncol. *14*, 217-224.

Elola,M.T., Chiesa,M.E., Alberti,A.F., Mordoh,J., and Fink,N.E. (2005). Galectin-1 receptors in different cell types. J Biomed. Sci. *12*, 13-29.

Elola,M.T., Wolfenstein-Todel,C., Troncoso,M.F., Vasta,G.R., and Rabinovich,G.A. (2007). Galectins: matricellular glycan-binding proteins linking cell adhesion, migration, and survival. Cell Mol. Life Sci. *64*, 1679-1700.

Eshkar Sebban,L., Ronen,D., Levartovsky,D., Elkayam,O., Caspi,D., Aamar,S., Amital,H., Rubinow,A., Golan,I., Naor,D., Zick,Y., and Golan,I. (2007). The Involvement of CD44 and Its Novel Ligand Galectin-8 in Apoptotic Regulation of Autoimmune Inflammation. J Immunol *179*, 1225-1235.

Farahat,M.N., Yanni,G., Poston,R., and Panayi,G.S. (1993). Cytokine expression in synovial membranes of patients with rheumatoid arthritis and osteoarthritis. Ann. Rheum. Dis. *52*, 870-875.

Farnworth,S.L., Henderson,N.C., Mackinnon,A.C., Atkinson,K.M., Wilkinson,T., Dhaliwal,K., Hayashi,K., Simpson,A.J., Rossi,A.G., Haslett,C., and Sethi,T. (2008). Galectin-3 Reduces the Severity of Pneumococcal Pneumonia by Augmenting Neutrophil Function. Am J Pathol *172*, 395-405.

Feldmann,M., Brennan,F.M., and Maini,R.N. (1996). Role of cytokines in rheumatoid arthritis. Annu. Rev. Immunol. *14:397-440.*, 397-440.

Feuk-Lagerstedt,E., Jordan,E.T., Leffler,H., Dahlgren,C., and Karlsson,A. (1999). Identification of CD66a and CD66b as the major galectin-3 receptor candidates in human neutrophils. J. Immunol. *163*, 5592-5598.

Filer, A. A molecular and functional comparison of synovial, bone marrow and dermal fibroblasts in rheumatoid and osteoarthritis. 2006. University of Birmingham.
Ref Type: Thesis/Dissertation

Filer,A., Bik,M., Parsonage,G.N., Fitton,J., Trebilcock,E., Howlett,K., Cook,M., Raza,K., Simmons,D.L., Thomas,A.M., Salmon,M., Scheel-Toellner,D., Lord,J.M., Rabinovich,G.A., and Buckley,C.D. (2009). Galectin 3 induces a distinctive pattern of cytokine and chemokine production in rheumatoid synovial fibroblasts via selective signaling pathways. Arthritis Rheum. *60*, 1604-1614.

Filer,A., Parsonage,G., Smith,E., Osborne,C., Thomas,A.M., Curnow,S.J., Rainger,G.E., Raza,K., Nash,G.B., Lord,J., Salmon,M., and Buckley,C.D. (2006a). Differential survival of leukocyte subsets mediated by synovial, bone marrow, and skin fibroblasts: site-specific versus activation-dependent survival of T cells and neutrophils. Arthritis Rheum. *54*, 2096-2108.

Filer,A., Pitzalis,C., and Buckley,C.D. (2006b). Targeting the stromal microenvironment in chronic inflammation. Curr. Opin. Pharmacol. *6*, 393-400.

Firestein,G.S., varo-Gracia,J.M., and Maki,R. (1990). Quantitative analysis of cytokine gene expression in rheumatoid arthritis. J Immunol. *144*, 3347-3353.

Firestein,G.S. and Zvaifler,N.J. (1987). Peripheral blood and synovial fluid monocyte activation in inflammatory arthritis. II. Low levels of synovial fluid and synovial tissue interferon suggest that gamma-interferon is not the primary macrophage activating factor. Arthritis Rheum. *30*, 864-871.

Firestein,G.S. and Zvaifler,N.J. (2002). How important are T cells in chronic rheumatoid synovitis?: II. T cell-independent mechanisms from beginning to end. Arthritis Rheum. *46*, 298-308.

Firestein,G.S. (2003). Evolving concepts of rheumatoid arthritis. Nature *423*, 356-361.

Fitton, J. Site Specific Responses of Fibroblasts to Galectin Stimulation. 2007. University of Birmingham.
Ref Type: Thesis/Dissertation

Forsman,H., Salomonsson,E., Onnheim,K., Karlsson,J., Bjorstad,A., Leffler,H., Bylund,J., Karlsson,A., and Dahlgren,C. (2008). The {beta}-galactoside binding immunomodulatory lectin galectin-3 reverses the desensitized state induced in neutrophils by the chemotactic peptide f-Met-Leu-Phe: role of reactive oxygen species generated by the NADPH-oxidase and inactivation of the agonist. Glycobiology *18*, 905-912.

Fossati,G., Bucknall,R.C., and Edwards,S.W. (2002). Insoluble and soluble immune complexes activate neutrophils by distinct activation mechanisms: changes in functional responses induced by priming with cytokines. Ann Rheum Dis *61*, 13-19.

Fossiez,F., Djossou,O., Chomarat,P., Flores-Romo,L., Ait-Yahia,S., Maat,C., Pin,J.J., Garrone,P., Garcia,E., Saeland,S., Blanchard,D., Gaillard,C., Das,M.B., Rouvier,E., Golstein,P., Banchereau,J., and Lebecque,S. (1996). T cell interleukin-17

induces stromal cells to produce proinflammatory and hematopoietic cytokines. J. Exp. Med. *183*, 2593-2603.

Foti,M., Granucci,F., and Ricciardi-Castagnoli,P. (2004). A central role for tissue-resident dendritic cells in innate responses. Trends in Immunology *25*, 650-654.

Fox,D.A. (2009). The role of T lymphocytes in rheumatoid arthritis. In Reumatoid arthritis, G.S.Firestein, G.S.Panayi, and F.A.Wollheim, eds. Oxford University Press), pp. 95-106.

Franz,J.K., Kolb,S.A., Hummel,K.M., Lahrtz,F., Neidhart,M., Aicher,W.K., Pap,T., Gay,R.E., Fontana,A., and Gay,S. (1998). Interleukin-16, produced by synovial fibroblasts, mediates chemoattraction for CD4+ T lymphocytes in rheumatoid arthritis. Eur. J Immunol. *28*, 2661-2671.

Fries,K.M., Blieden,T., Looney,R.J., Sempowski,G.D., Silvera,M.R., Willis,R.A., and Phipps,R.P. (1994). Evidence of fibroblast heterogeneity and the role of fibroblast subpopulations in fibrosis. Clin. Immunol. Immunopathol. *72*, 283-292.

Frol'ova,L., Smetana,K., Jr., Borovska,D., Kitanovicova,A., Klimesova,K., Janatkova,I., Malickova,K., Lukas,M., Drastich,P., Benes,Z., Tuckova,L., Manning,J.C., Andre,S., Gabius,H.J., and Tlaskalova-Hogenova,H. (2009). Detection of galectin-3 in patients with inflammatory bowel diseases: new serum marker of active forms of IBD? Inflamm. Res.

Fu,Y.X., Huang,G., Wang,Y., and Chaplin,D.D. (1998). B lymphocytes induce the formation of follicular dendritic cell clusters in a lymphotoxin alpha-dependent fashion. J. Exp. Med. *187*, 1009-1018.

Fujisawa,K., Aono,H., Hasunuma,T., Yamamoto,K., Mita,S., and Nishioka,K. (1996). Activation of transcription factor NF-kappa B in human synovial cells in response to tumor necrosis factor alpha. Arthritis Rheum. *39*, 197-203.

Fukumori,T., Takenaka,Y., Yoshii,T., Kim,H.R.C., Hogan,V., Inohara,H., Kagawa,S., and Raz,A. (2003a). CD29 and CD7 Mediate Galectin-3-Induced Type II T-Cell Apoptosis. Cancer Res *63*, 8302-8311.

Fukumori,T., Takenaka,Y., Yoshii,T., Kim,H.R.C., Hogan,V., Inohara,H., Kagawa,S., and Raz,A. (2003). CD29 and CD7 Mediate Galectin-3-Induced Type II T-Cell Apoptosis. Cancer Res *63*, 8302-8311.

Fukuoka,M., Ogino,Y., Sato,H., Ohta,T., and Komoriya,K. (1998). Regulation of RANTES and IL-8 production in normal human dermal fibroblasts by active vitamin D3 (tacalcitol). Br. J Pharmacol. *124*, 1433-1438.

Gaber,T., Dziurla,R., Tripmacher,R., Burmester,G.R., and Buttgereit,F. (2005). Hypoxia inducible factor (HIF) in rheumatology: low O2! See what HIF can do! Ann. Rheum. Dis. *64*, 971-980.

Gao,J.X. and Issekutz,A.C. (1997). The beta 1 integrin, very late activation antigen-4 on human neutrophils can contribute to neutrophil migration through connective tissue fibroblast barriers. Immunology. *90*, 448-454.

Garlanda,C. and Dejana,E. (1997). Heterogeneity of endothelial cells. Specific markers. Arterioscler. Thromb. Vasc. Biol. *17*, 1193-1202.

Genin,P., Algarte,M., Roof,P., Lin,R., and Hiscott,J. (2000). Regulation of RANTES Chemokine Gene Expression Requires Cooperativity Between NF-{kappa}B and IFN-Regulatory Factor Transcription Factors. J Immunol *164*, 5352-5361.

Georganas,C., Liu,H., Perlman,H., Hoffmann,A., Thimmapaya,B., and Pope,R.M. (2000). Regulation of IL-6 and IL-8 expression in rheumatoid arthritis synovial fibroblasts: the dominant role for NF-kappa B but not C/EBP beta or c-Jun. J Immunol. *165*, 7199-7206.

Ginis,I. and Faller,D.V. (1997). Protection from apoptosis in human neutrophils is determined by the surface of adhesion. Am J Physiol Cell Physiol *272*, C295-C309.

Goldberg,M., Nadiv,O., Luknar-Gabor,N., Agar,G., Beer,Y., and Katz,Y. (2009). Synergism between tumor necrosis factor alpha and interleukin-17 to induce IL-23 p19 expression in fibroblast-like synoviocytes. Mol. Immunol. *46*, 1854-1859.

Goldring,S.R. and Gravallese,E.M. (2000). Mechanisms of bone loss in inflammatory arthritis: diagnosis and therapeutic implications. Arthritis Res. *2*, 33-37.

Gombert,W., Borthwick,N.J., Wallace,D.L., Hyde,H., Bofill,M., Pilling,D., Beverley,P.C., Janossy,G., Salmon,M., and Akbar,A.N. (1996). Fibroblasts prevent apoptosis of IL-2-deprived T cells without inducing proliferation: a selective effect on Bcl-XL expression. Immunology. *89*, 397-404.

Gopalkrishnan,R.V., Roberts,T., Tuli,S., Kang,D., Christiansen,K.A., and Fisher,P.B. (2000). Molecular characterization of prostate carcinoma tumor antigen-1, PCTA-1, a human galectin-8 related gene. Oncogene. *19*, 4405-4416.

Goronzy,J.J. and Weyand,C.M. (2003). Aging, autoimmunity and arthritis: T-cell senescence and contraction of T-cell repertoire diversity - catalysts of autoimmunity and chronic inflammation. Arthritis Res. Ther. *5*, 225-234.

Goronzy,J.J., Zettl,A., and Weyand,C.M. (1998). T cell receptor repertoire in rheumatoid arthritis. Int. Rev. Immunol. *17*, 339-363.

Gregersen,P.K., Plenge,R.M., and Gulko,P.S. (2006). Genetics of rheumatoid arthrits. In Rheumatoid arthritis, G.S.Firestein, G.S.Panayi, and F.A.Wollheim, eds. Oxford University Press), pp. 4-14.

Gregersen,P.K., Silver,J., and Winchester,R.J. (1987). The shared epitope hypothesis. An approach to understanding the molecular genetics of susceptibility to rheumatoid arthritis. Arthritis Rheum. *30*, 1205-1213.

Guo,H.B., Nairn,A., Harris,K., Randolph,M., Alvarez-Manilla,G., Moremen,K., and Pierce,M. (2008). Loss of expression of N-acetylglucosaminyltransferase Va results in altered gene expression of glycosyltransferases and galectins. FEBS Lett. *%20;582*, 527-535.

Gupta,S.K., Masinick,S., Garrett,M., and Hazlett,L.D. (1997). Pseudomonas aeruginosa lipopolysaccharide binds galectin-3 and other human corneal epithelial proteins. Infect. Immun. *65*, 2747-2753.

Hadari,Y.R., Arbel-Goren,R., Levy,Y., Amsterdam,A., Alon,R., Zakut,R., and Zick,Y. (2000). Galectin-8 binding to integrins inhibits cell adhesion and induces apoptosis. J Cell Sci *113*, 2385-2397.

Hahn,H.P., Pang,M., He,J., Hernandez,J.D., Yang,R.Y., Li,L.Y., Wang,X., Liu,F.T., and Baum,L.G. (2004). Galectin-1 induces nuclear translocation of endonuclease G in caspase- and cytochrome c-independent T cell death. Cell Death Differ *11*, 1277-1286.

Harjacek,M., az-Cano,S., De Miguel,M., Wolfe,H., Maldonado,C.A., and Rabinovich,G.A. (2001). Expression of galectins-1 and -3 correlates with defective mononuclear cell apoptosis in patients with juvenile idiopathic arthritis. The Journal of Rheumatology *28*, 1914-1922.

Harrison,B.J. (2002). Influence of cigarette smoking on disease outcome in rheumatoid arthritis. Curr. Opin. Rheumatol. *14*, 93-97.

Hastings, W., Greer, A., Kuchroo, V., Hafler, D. A., Kent, S., and Anderson, D. Dysregulation of TIM-3-galectin-9 pathway in type I diabetic T cells. Clin.Immunol. 123[Supplement 1], S21. 2007.
Ref Type: Abstract

Henderson,N.C., Mackinnon,A.C., Farnworth,S.L., Poirier,F., Russo,F.P., Iredale,J.P., Haslett,C., Simpson,K.J., and Sethi,T. (2006). Galectin-3 regulates myofibroblast activation and hepatic fibrosis. Proc. Natl. Acad. Sci. U. S. A. *103*, 5060-5065.

Henderson,N.C., Mackinnon,A.C., Farnworth,S.L., Kipari,T., Haslett,C., Iredale,J.P., Liu,F.T., Hughes,J., and Sethi,T. (2008). Galectin-3 Expression and Secretion Links Macrophages to the Promotion of Renal Fibrosis. Am J Pathol *172*, 288-298.

Hernandez,J.D., Nguyen,J.T., He,J., Wang,W., Ardman,B., Green,J.M., Fukuda,M., and Baum,L.G. (2006). Galectin-1 Binds Different CD43 Glycoforms to Cluster CD43 and Regulate T Cell Death. J Immunol *177*, 5328-5336.

Hilbert,N., Schiller,J., Arnhold,J., and Arnold,K. (2002). Cartilage Degradation by Stimulated Human Neutrophils: Elastase Is Mainly Responsible for Cartilage Damage. Bioorganic Chemistry *30*, 119-132.

Hirabayashi,J., Hashidate,T., Arata,Y., Nishi,N., Nakamura,T., Hirashima,M., Urashima,T., Oka,T., Futai,M., Muller,W.E., Yagi,F., and Kasai,K. (2002).

Oligosaccharide specificity of galectins: a search by frontal affinity chromatography. Biochim. Biophys. Acta. *%19;1572*, 232-254.

Hirashima,M., Kashio,Y., Nishi,N., Yamauchi,A., Imaizumi,T.A., Kageshita,T., Saita,N., and Nakamura,T. (2004). Galectin-9 in physiological and pathological conditions. Glycoconj. J. *19*, 593-600.

Hokama,A., Mizoguchi,E., and Mizoguchi,A. (2008). Roles of galectins in inflammatory bowel disease. World J Gastroenterol. *14*, 5133-5137.

Hokama,A., Mizoguchi,E., Sugimoto,K., Shimomura,Y., Tanaka,Y., Yoshida,M., Rietdijk,S.T., de Jong,Y.P., Snapper,S.B., Terhorst,C., Blumberg,R.S., and Mizoguchi,A. (2004). Induced reactivity of intestinal CD4(+) T cells with an epithelial cell lectin, galectin-4, contributes to exacerbation of intestinal inflammation. Immunity. *20*, 681-693.

Honjo,Y., Nangia-Makker,P., Inohara,H., and Raz,A. (2001). Down-regulation of galectin-3 suppresses tumorigenicity of human breast carcinoma cells. Clin. Cancer Res. *7*, 661-668.

Horsfall,A.C., Butler,D.M., Marinova,L., Warden,P.J., Williams,R.O., Maini,R.N., and Feldmann,M. (1997). Suppression of collagen-induced arthritis by continuous administration of IL-4. J Immunol *159*, 5687-5696.

Hotta,K., Funahashi,T., Matsukawa,Y., Takahashi,M., Nishizawa,H., Kishida,K., Matsuda,M., Kuriyama,H., Kihara,S., Nakamura,T., Tochino,Y., Bodkin,N.L., Hansen,B.C., and Matsuzawa,Y. (2001). Galectin-12, an Adipose-expressed Galectin-like Molecule Possessing Apoptosis-inducing Activity. J. Biol. Chem. *276*, 34089-34097.

Hoyer,K.K., Pang,M., Gui,D., Shintaku,I.P., Kuwabara,I., Liu,F.T., Said,J.W., Baum,L.G., and Teitell,M.A. (2004). An anti-apoptotic role for galectin-3 in diffuse large B-cell lymphomas. Am. J. Pathol. *164*, 893-902.

Hsu,D.K. and Liu,F.T. (2004). Regulation of cellular homeostasis by galectins. Glycoconj. J. *19*, 507-515.

Hsu,D.K., Yang,R.Y., and Liu,F.T. (2006). Galectins in apoptosis. Methods Enzymol. *417:256-73.*, 256-273.

Hsu,D.K., Yang,R.Y., Pan,Z., Yu,L., Salomon,D.R., Fung-Leung,W.P., and Liu,F.T. (2000). Targeted disruption of the galectin-3 gene results in attenuated peritoneal inflammatory responses. Am. J Pathol. *156*, 1073-1083.

Hu,M., Lin,X., Du,Q., Miller,E.J., Wang,P., and Simms,H.H. (2005). Regulation of polymorphonuclear leukocyte apoptosis: role of lung endothelium-epithelium bilayer transmigration. Am J Physiol Lung Cell Mol. Physiol. *288*, L266-L274.

Huber,L.C., Distler,O., Tarner,I., Gay,R.E., Gay,S., and Pap,T. (2006). Synovial fibroblasts: key players in rheumatoid arthritis. Rheumatology. (Oxford). *45*, 669-675.

Huflejt,M.E. and Leffler,H. (2004). Galectin-4 in normal tissues and cancer. Glycoconj. J. *20*, 247-255.

Hughes,R.C. (1999). Secretion of the galectin family of mammalian carbohydrate-binding proteins. Biochim. Biophys. Acta. *1473*, 172-185.

Hummel,K.M., Petrow,P.K., Franz,J.K., Muller-Ladner,U., Aicher,W.K., Gay,R.E., Bromme,D., and Gay,S. (1998). Cysteine proteinase cathepsin K mRNA is expressed in synovium of patients with rheumatoid arthritis and is detected at sites of synovial bone destruction. J. Rheumatol. *25*, 1887-1894.

Hwang,S.Y., Kim,J.Y., Kim,K.W., Park,M.K., Moon,Y., Kim,W.U., and Kim,H.Y. (2004). IL-17 induces production of IL-6 and IL-8 in rheumatoid arthritis synovial fibroblasts via NF-kappaB- and PI3-kinase/Akt-dependent pathways. Arthritis Res. Ther. *6*, R120-R128.

Hyrich,K.L. and Inman,R.D. (2001). Infectious agents in chronic rheumatic diseases. Curr. Opin. Rheumatol. *13*, 300-304.

Igawa,K., Satoh,T., Hirashima,M., and Yokozeki,H. (2006). Regulatory mechanisms of galectin-9 and eotaxin-3 synthesis in epidermal keratinocytes: possible involvement of galectin-9 in dermal eosinophilia of Th1-polarized skin inflammation. Allergy. *61*, 1385-1391.

Ilarregui,J.M., Bianco,G.A., Toscano,M.A., and Rabinovich,G.A. (2005). The coming of age of galectins as immunomodulatory agents: impact of these carbohydrate binding proteins in T cell physiology and chronic inflammatory disorders. Ann. Rheum. Dis. *64 Suppl 4:iv96-103.*, iv96-103.

Imaizumi,T., Kumagai,M., Sasaki,N., Kurotaki,H., Mori,F., Seki,M., Nishi,N., Fujimoto,K., Tanji,K., Shibata,T., Tamo,W., Matsumiya,T., Yoshida,H., Cui,X.F., Takanashi,S., Hanada,K., Okumura,K., Yagihashi,S., Wakabayashi,K., Nakamura,T., Hirashima,M., and Satoh,K. (2002). Interferon-{gamma} stimulates the expression of galectin-9 in cultured human endothelial cells. J Leukoc Biol *72*, 486-491.

Imaizumi,T., Yoshida,H., Nishi,N., Sashinami,H., Nakamura,T., Hirashima,M., Ohyama,C., Itoh,K., and Satoh,K. (2007). Double-stranded RNA induces galectin-9 in vascular endothelial cells: involvement of TLR3, PI3K, and IRF3 pathway. Glycobiology *17*, 12C-15.

Inohara,H., Akahani,S., and Raz,A. (1998). Galectin-3 stimulates cell proliferation. Exp. Cell Res. *245*, 294-302.

IIrie,A., Yamauchi,A., Kontani,K., Kihara,M., Liu,D., Shirato,Y., Seki,M., Nishi,N., Nakamura,T., Yokomise,H., and Hirashima,M. (2005). Galectin-9 as a Prognostic

Factor with Antimetastatic Potential in Breast Cancer. Clin Cancer Res *11*, 2962-2968.

Iurisci,I., Tinari,N., Natoli,C., Angelucci,D., Cianchetti,E., and Iacobelli,S. (2000). Concentrations of galectin-3 in the sera of normal controls and cancer patients. Clin. Cancer Res. *6*, 1389-1393.

Janelle-Montcalm,A., Boileau,C., Poirier,F., Pelletier,J.P., Guevremont,M., Duval,N., Martel-Pelletier,J., and Reboul,P. (2007). Extracellular localization of galectin-3 has a deleterious role in joint tissues. Arthritis Res Ther. *9*, R20.

Jeng,K.C., Frigeri,L.G., and Liu,F.T. (1994). An endogenous lectin, galectin-3 (epsilon BP/Mac-2), potentiates IL-1 production by human monocytes. Immunol Lett. *42*, 113-116.

Jiang,H.R., Al Rasebi,Z., Mensah-Brown,E., Shahin,A., Xu,D., Goodyear,C.S., Fukada,S.Y., Liu,F.T., Liew,F.Y., and Lukic,M.L. (2009). Galectin-3 Deficiency Reduces the Severity of Experimental Autoimmune Encephalomyelitis. J Immunol *182*, 1167-1173.

Jones,P.A. and Baylin,S.B. (2007). The epigenomics of cancer. Cell. *128*, 683-692.

Joo,H.G., Goedegebuure,P.S., Sadanaga,N., Nagoshi,M., von Bernstorff,W., and Eberlein,T.J. (2001). Expression and function of galectin-3, a {beta}-galactoside-binding protein in activated T lymphocytes. J Leukoc Biol *69*, 555-564.

Joosten,L.A., Koenders,M.I., Smeets,R.L., Heuvelmans-Jacobs,M., Helsen,M.M., Takeda,K., Akira,S., Lubberts,E., van de Loo,F.A., and van den Berg,W.B. (2003). Toll-like receptor 2 pathway drives streptococcal cell wall-induced joint inflammation: critical role of myeloid differentiation factor 88. J Immunol. *171*, 6145-6153.

Jordan,N.J., Watson,M.L., Yoshimura,T., and Westwick,J. (1996). Differential effects of protein kinase C inhibitors on chemokine production in human synovial fibroblasts. Br. J Pharmacol. *117*, 1245-1253.

Kadrofske,M.M., Openo,K.P., and Wang,J.L. (1998). The human LGALS3 (galectin-3) gene: determination of the gene structure and functional characterization of the promoter. Arch. Biochem. Biophys. *349*, 7-20.

Kageshita,T., Kashio,Y., Yamauchi,A., Seki,M., Abedin,M.J., Nishi,N., Shoji,H., Nakamura,T., Ono,T., and Hirashima,M. (2002). Possible role of galectin-9 in cell aggregation and apoptosis of human melanoma cell lines and its clinical significance. Int. J. Cancer. *%20;99*, 809-816.

Kariko,K., Ni,H., Capodici,J., Lamphier,M., and Weissman,D. (2004). mRNA is an endogenous ligand for Toll-like receptor 3. J Biol. Chem. *279*, 12542-12550.

Karlsson,A., Christenson,K., Matlak,M., Bjorstad,A., Brown,K.L., Telemo,E., Salomonsson,E., Leffler,H., and Bylund,J. (2009). Galectin-3 functions as an

opsonin and enhances the macrophage clearance of apoptotic neutrophils. Glycobiology *19*, 16-20.

Karlsson,A., Follin,P., Leffler,H., and Dahlgren,C. (1998). Galectin-3 Activates the NADPH-Oxidase in Exudated but not Peripheral Blood Neutrophils. Blood *91*, 3430-3438.

Karouzakis, E. and et al. Genomic hypomethylation of rheumatoid arthritis synovial fibroblasts. Arthritis Rheum. 56[suppl.], S317. 2007.
Ref Type: Abstract

Karouzakis,E., Gay,R.E., Gay,S., and Neidhart,M. (2009). Epigenetic control in rheumatoid arthritis synovial fibroblasts. Nat. Rev. Rheumatol. *5*, 266-272.

Karouzakis, E., Gay, R. E., Kolling, C., Gay, S., and Neidhart, M. Epigentic profile of gene expression in normal and rheumatoid arthrits synovial fibroblasts. Arthritis Rheum. 58[suppl.], S514. 2008.
Ref Type: Abstract

Kashio,Y., Nakamura,K., Abedin,M.J., Seki,M., Nishi,N., Yoshida,N., Nakamura,T., and Hirashima,M. (2003). Galectin-9 Induces Apoptosis Through the Calcium-Calpain-Caspase-1 Pathway. J Immunol *170*, 3631-3636.

Katz,Y., Nadiv,O., and Beer,Y. (2001). Interleukin-17 enhances tumor necrosis factor alpha-induced synthesis of interleukins 1,6, and 8 in skin and synovial fibroblasts: a possible role as a "fine-tuning cytokine" in inflammation processes. Arthritis Rheum. *44*, 2176-2184.

Kaur,S., Katsoulidis,E., and Platanias,L.C. (2008). Akt and mRNA translation by interferons. Cell Cycle. *7*, 2112-2116.

Kawane,K., Ohtani,M., Miwa,K., Kizawa,T., Kanbara,Y., Yoshioka,Y., Yoshikawa,H., and Nagata,S. (2006). Chronic polyarthritis caused by mammalian DNA that escapes from degradation in macrophages. Nature. *443*, 998-1002.

Kettritz,R., Gaido,M.L., Haller,H., Luft,F.C., Jennette,C.J., and Falk,R.J. (1998). Interleukin-8 delays spontaneous and tumor necrosis factor-[agr]-mediated apoptosis of human neutrophils. Kidney Int *53*, 84-91.

Keyszer,G., Redlich,A., Haupl,T., Zacher,J., Sparmann,M., Engethum,U., Gay,S., and Burmester,G.R. (1998). Differential expression of cathepsins B and L compared with matrix metalloproteinases and their respective inhibitors in rheumatoid arthritis and osteoarthritis: a parallel investigation by semiquantitative reverse transcriptase-polymerase chain reaction and immunohistochemistry. Arthritis Rheum. *41*, 1378-1387.

Kim,H.J., Krenn,V., Steinhauser,G., and Berek,C. (1999a). Plasma Cell Development in Synovial Germinal Centers in Patients with Rheumatoid and Reactive Arthritis. J Immunol *162*, 3053-3062.

Kim,H.R.C., Lin,H.M., Biliran,H., and Raz,A. (1999b). Cell Cycle Arrest and Inhibition of Anoikis by Galectin-3 in Human Breast Epithelial Cells. Cancer Res *59*, 4148-4154.

Kim,Y.I., Logan,J.W., Mason,J.B., and Roubenoff,R. (1996). DNA hypomethylation in inflammatory arthritis: reversal with methotrexate. J Lab Clin Med. *128*, 165-172.

Kinne,R.W., Kunisch,E., Beensen,V., Zimmermann,T., Emmrich,F., Petrow,P., Lungershausen,W., Hein,G., Braun,R.K., Foerster,M., Kroegel,C., Winter,R., Liesaus,E., Fuhrmann,R.A., Roth,A., Claussen,U., and Liehr,T. (2003). Synovial fibroblasts and synovial macrophages from patients with rheumatoid arthritis and other inflammatory joint diseases show chromosomal aberrations. Genes Chromosomes. Cancer. *38*, 53-67.

Kinne,R.W., Stuhlmuller,B., Palombo-Kinne,E., and Burmester,G.R. (2006). The role of macrophages in rheumatoid arthritis. In Rheumatoid arthritis, G.S.Firestein, G.S.Panayi, and F.A.Wollheim, eds. Oxford University Press), pp. 55-76.

Kinne,R., Stuhlmuller,B., and Burmester,G.R. (2007). Cells of the synovium in rheumatoid arthritis. Macrophages. Arthritis Research & Therapy *9*, 224.

Kinne,R.W., Palombo-Kinne,E., and Emmrich,F. (1997). T-cells in the pathogenesis of rheumatoid arthritis Villains or accomplices? Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease *1360*, 109-141.

Klareskog,L., Padyukov,L., and Alfredsson,L. (2007). Smoking as a trigger for inflammatory rheumatic diseases. Curr. Opin. Rheumatol. *19*, 49-54.

Klimiuk,P.A., Goronzy,J.J., Bjor,n.J., Beckenbaugh,R.D., and Weyand,C.M. (1997). Tissue cytokine patterns distinguish variants of rheumatoid synovitis. Am. J Pathol. *151*, 1311-1319.

Klimiuk,P.A., Sierakowski,S., Latosiewicz,R., Skowronski,J., Cylwik,J.P., Cylwik,B., and Chwiecko,J. (2005). Histological patterns of synovitis and serum chemokines in patients with rheumatoid arthritis. The Journal of Rheumatology *32*, 1666-1672.

Kobayashi,S.D., Voyich,J.M., Whitney,A.R., and DeLeo,F.R. (2005). Spontaneous neutrophil apoptosis and regulation of cell survival by granulocyte macrophage-colony stimulating factor. J Leukoc Biol *78*, 1408-1418.

Kobayashi,T., Okamoto,K., Kobata,T., Hasunuma,T., Kato,T., Hamada,H., and Nishioka,K. (2000). Differential regulation of Fas-mediated apoptosis of rheumatoid synoviocytes by tumor necrosis factor alpha and basic fibroblast growth factor is associated with the expression of apoptosis-related molecules. Arthritis Rheum. *43*, 1106-1114.

Koguchi,K., Anderson,D.E., Yang,L., O'Connor,K.C., Kuchroo,V.K., and Hafler,D.A. (2006). Dysregulated T cell expression of TIM3 in multiple sclerosis. J. Exp. Med. *203*, 1413-1418.

Kondoh,N., Wakatsuki,T., Ryo,A., Hada,A., Aihara,T., Horiuchi,S., Goseki,N., Matsubara,O., Takenaka,K., Shichita,M., Tanaka,K., Shuda,M., and Yamamoto,M. (1999). Identification and characterization of genes associated with human hepatocellular carcinogenesis. Cancer Res. *59*, 4990-4996.

Kontoyiannis,D. and Kollias,G. (2000). Fibroblast biology: Synovial fibroblasts in rheumatoid arthritis - leading role or chorus line? Arthritis Res *2*, 342-343.

Konttinen,Y.T., Li,T.F., Hukkanen,M., Ma,J., Xu,J.W., and Virtanen,I. (2000). Fibroblast biology. Signals targeting the synovial fibroblast in arthritis. Arthritis Res. *2*, 348-355.

Konttinen,Y.T., Ainola,M., Valleala,H., Ma,J., Ida,H., Mandelin,J., Kinne,R.W., Santavirta,S., Sorsa,T., Lopez-Otin,C., and Takagi,M. (1999). Analysis of 16 different matrix metalloproteinases (MMP-1 to MMP-20) in the synovial membrane: different profiles in trauma and rheumatoid arthritis. Ann Rheum Dis *58*, 691-697.

Kotake,S., Schumacher,H.R., Jr., Yarboro,C.H., Arayssi,T.K., Pando,J.A., Kanik,K.S., Gourley,M.F., Klippel,J.H., and Wilder,R.L. (1997). In vivo gene expression of type 1 and type 2 cytokines in synovial tissues from patients in early stages of rheumatoid, reactive, and undifferentiated arthritis. Proc. Assoc. Am. Physicians. *109*, 286-301.

Kotake,S., Udagawa,N., Takahashi,N., Matsuzaki,K., Itoh,K., Ishiyama,S., Saito,S., Inoue,K., Kamatani,N., Gillespie,M.T., Martin,T.J., and Suda,T. (1999). IL-17 in synovial fluids from patients with rheumatoid arthritis is a potent stimulator of osteoclastogenesis. J. Clin. Invest. *103*, 1345-1352.

Kotzin,B.L., Falta,M.T., Crawford,F., Rosloniec,E.F., Bill,J., Marrack,P., and Kappler,J. (2000). Use of soluble peptide-DR4 tetramers to detect synovial T cells specific for cartilage antigens in patients with rheumatoid arthritis. Proc. Natl. Acad. Sci. U. S. A. *97*, 291-296.

Kouzarides,T. (2007). Chromatin Modifications and Their Function. Cell *128*, 693-705.

Kubach,J., Lutter,P., Bopp,T., Stoll,S., Becker,C., Huter,E., Richter,C., Weingarten,P., Warger,T., Knop,J., Mullner,S., Wijdenes,J., Schild,H., Schmitt,E., and Jonuleit,H. (2007). Human CD4+CD25+ regulatory T cells: proteome analysis identifies galectin-10 as a novel marker essential for their anergy and suppressive function. Blood blood-2007.

Kunisch,E., Gandesiri,M., Fuhrmann,R., Roth,A., Winter,R., and Kinne,R.W. (2007). Predominant activation of MAP kinases and pro-destructive/pro-inflammatory features by TNF alpha in early-passage synovial fibroblasts via TNF receptor-1: failure of p38 inhibition to suppress matrix metalloproteinase-1 in rheumatoid arthritis. Ann Rheum Dis. *66*, 1043-1051.

Kuwabara,I. and Liu,F.T. (1996). Galectin-3 promotes adhesion of human neutrophils to laminin. J Immunol *156*, 3939-3944.

Kuwabara,I., Kuwabara,Y., Yang,R.Y., Schuler,M., Green,D.R., Zuraw,B.L., Hsu,D.K., and Liu,F.T. (2002). Galectin-7 (PIG1) Exhibits Pro-apoptotic Function through JNK Activation and Mitochondrial Cytochrome c Release. J. Biol. Chem. *277*, 3487-3497.

Kyburz,D., Rethage,J., Seibl,R., Lauener,R., Gay,R.E., Carson,D.A., and Gay,S. (2003). Bacterial peptidoglycans but not CpG oligodeoxynucleotides activate synovial fibroblasts by toll-like receptor signaling. Arthritis Rheum. *48*, 642-650.

La,M., Cao,T.V., Cerchiaro,G., Chilton,K., Hirabayashi,J., Kasai,K.i., Oliani,S.M., Chernajovsky,Y., and Perretti,M. (2003). A Novel Biological Activity for Galectin-1: Inhibition of Leukocyte-Endothelial Cell Interactions in Experimental Inflammation. Am J Pathol *163*, 1505-1515.

Laan,M., Prause,O., Miyamoto,M., Sjostrand,M., Hytonen,A.M., Kaneko,T., Lotvall,J., and Linden,A. (2003). A role of GM-CSF in the accumulation of neutrophils in the airways caused by IL-17 and TNF-alpha. Eur. Respir. J. *21*, 387-393.

Lafyatis,R., Remmers,E.F., Roberts,A.B., Yocum,D.E., Sporn,M.B., and Wilder,R.L. (1989). Anchorage-independent growth of synoviocytes from arthritic and normal joints. Stimulation by exogenous platelet-derived growth factor and inhibition by transforming growth factor-beta and retinoids. J. Clin. Invest. *83*, 1267-1276.

Lahm,H., Andre,S., Hoeflich,A., Kaltner,H., Siebert,H.C., Sordat,B., der Lieth,C.W., Wolf,E., and Gabius,H.J. (2004). Tumor galectinology: insights into the complex network of a family of endogenous lectins. Glycoconj. J. *20*, 227-238.

Lahm,H., Hoeflich,A., Andre,S., Sordat,B., Kaltner,H., Wolf,E., and Gabius,H.J. (2000). Gene expression of galectin-9/ecalectin, a potent eosinophil chemoattractant, and/or the insertional isoform in human colorectal carcinoma cell lines and detection of frame-shift mutations for protein sequence truncations in the second functional lectin domain. Int. J Oncol. *17*, 519-524.

Lally,F., Smith,E., Filer,A., Stone,M.A., Shaw,J.S., Nash,G.B., Buckley,C.D., and Rainger,G.E. (2005). A novel mechanism of neutrophil recruitment in a coculture model of the rheumatoid synovium. Arthritis Rheum. *52*, 3460-3469.

Lau,K.S., Partridge,E.A., Grigorian,A., Silvescu,C.I., Reinhold,V.N., Demetriou,M., and Dennis,J.W. (2007). Complex N-glycan number and degree of branching cooperate to regulate cell proliferation and differentiation. Cell. *129*, 123-134.

Lee,A., Whyte,M.K., and Haslett,C. (1993). Inhibition of apoptosis and prolongation of neutrophil functional longevity by inflammatory mediators. J Leukoc Biol *54*, 283-288.

Lee,A.H., Hong,J.H., and Seo,Y.S. (2000). Tumour necrosis factor-alpha and interferon-gamma synergistically activate the RANTES promoter through nuclear factor kappaB and interferon regulatory factor 1 (IRF-1) transcription factors. Biochem. J. *350 Pt 1:131-8.*, 131-138.

Lee,D.M. and Weinblatt,M.E. (2001). Rheumatoid arthritis. The Lancet *358*, 903-911.

Lee,Y.J., Kang,S.W., Song,J.K., Park,J.J., Bae,Y.D., Lee,E.Y., Lee,E.B., and Song,Y.W. (2007). Serum galectin-3 and galectin-3 binding protein levels in Behcet's disease and their association with disease activity. Clin. Exp. Rheumatol. *25*, S41-S45.

Leffler,H., Carlsson,S., Hedlund,M., Qian,Y., and Poirier,F. (2004). Introduction to galectins. Glycoconj. J. *19*, 433-440.

Li,X. and Makarov,S.S. (2006). An essential role of NF-{kappa}B in the "tumor-like" phenotype of arthritic synoviocytes. PNAS *103*, 17432-17437.

Lin,H.M., Moon,B.K., Yu,F., and Kim,H.R. (2000). Galectin-3 mediates genistein-induced G(2)/M arrest and inhibits apoptosis. Carcinogenesis. *21*, 1941-1945.

Lindhout,E., van Eijk,M., van Pel,M., Lindeman,J., Dinant,H.J., and de Groot,C. (1999). Fibroblast-Like Synoviocytes from Rheumatoid Arthritis Patients Have Intrinsic Properties of Follicular Dendritic Cells. J Immunol *162*, 5949-5956.

Lipkowitz,M.S., Leal-Pinto,E., Cohen,B.E., and Abramson,R.G. (2004). Galectin 9 is the sugar-regulated urate transporter/channel UAT. Glycoconj. J. *19*, 491-498.

Lippert,E., Falk,W., Bataille,F., Kaehne,T., Naumann,M., Goeke,M., Herfarth,H., Schoelmerich,J., and Rogler,G. (2007). Soluble galectin-3 is a strong, colonic epithelial-cell-derived, lamina propria fibroblast-stimulating factor. Gut. *56*, 43-51.

Liu,F.T., Patterson,R.J., and Wang,J.L. (2002). Intracellular functions of galectins. Biochim. Biophys. Acta. *%19;1572*, 263-273.

Liu,F.T. and Rabinovich,G.A. (2005). Galectins as modulators of tumour progression. Nat. Rev. Cancer. *5*, 29-41.

Liu,S.D., Tomassian,T., Bruhn,K.W., Miller,J.F., Poirier,F., and Miceli,M.C. (2009). Galectin-1 tunes TCR binding and signal transduction to regulate CD8 burst size. J. Immunol. *182*, 5283-5295.

Liu,S.D., Whiting,C.C., Tomassian,T., Pang,M., Bissel,S.J., Baum,L.G., Mossine,V.V., Poirier,F., Huflejt,M.E., and Miceli,M.C. (2008). Endogenous galectin-1 enforces class I-restricted TCR functional fate decisions in thymocytes. Blood *112*, 120-130.

Liu,Z.Q., Deng,G.M., Foster,S., and Tarkowski,A. (2001). Staphylococcal peptidoglycans induce arthritis. Arthritis Res. *3*, 375-380.

Lopez,E., del Pozo,V., Miguel,T., Sastre,B., Seoane,C., Civantos,E., Llanes,E., Baeza,M.L., Palomino,P., Cardaba,B., Gallardo,S., Manzarbeitia,F., Zubeldia,J.M., and Lahoz,C. (2006). Inhibition of Chronic Airway Inflammation and Remodeling by Galectin-3 Gene Therapy in a Murine Model. J Immunol *176*, 1943-1950.

Lubberts,E., Koenders,M.I., and van den Berg,W.B. (2005). The role of T-cell interleukin-17 in conducting destructive arthritis: lessons from animal models. Arthritis Res. Ther. *7*, 29-37.

Lukyanov,P., Furtak,V., and Ochieng,J. (2005). Galectin-3 interacts with membrane lipids and penetrates the lipid bilayer. Biochem. Biophys. Res. Commun. *338*, 1031-1036.

Lundstrom,E., Kallberg,H., Alfredsson,L., Klareskog,L., and Padyukov,L. (2009). Gene-environment interaction between the DRB1 shared epitope and smoking in the risk of anti-citrullinated protein antibody-positive rheumatoid arthritis: All alleles are important. Arthritis Rheum. *60*, 1597-1603.

Mackinnon,A.C., Farnworth,S.L., Hodkinson,P.S., Henderson,N.C., Atkinson,K.M., Leffler,H., Nilsson,U.J., Haslett,C., Forbes,S.J., and Sethi,T. (2008). Regulation of Alternative Macrophage Activation by Galectin-3. J Immunol *180*, 2650-2658.

Madsen,P., Rasmussen,H.H., Flint,T., Gromov,P., Kruse,T.A., Honorθ,B., Vorum,H., and Celis,J.E. (1995). Cloning, Expression, and Chromosome Mapping of Human Galectin-7. J. Biol. Chem. *270*, 5823-5829.

Mageed,R.A., Kirwan,J.R., Thompson,P.W., McCarthy,D.A., and Holborow,E.J. (1991). Characterisation of the size and composition of circulating immune complexes in patients with rheumatoid arthritis. Ann Rheum Dis. *50*, 231-236.

Magnaldo,T., Fowlis,D., and Darmon,M. (1998). Galectin-7, a marker of all types of stratified epithelia. Differentiation. *63*, 159-168.

Makishi,S., Okudaira,T., Ishikawa,C., Sawada,S., Watanabe,T., Hirashima,M., Sunakawa,H., and Mori,N. (2008). A modified version of galectin-9 induces cell cycle arrest and apoptosis of Burkitt and Hodgkin lymphoma cells. Br. J Haematol. *142*, 583-594.

Marshak-Rothstein,A. (2006). Toll-like receptors in systemic autoimmune disease. Nat. Rev. Immunol. *6*, 823-835.

Marshak-Rothstein,A. and Rifkin,I.R. (2007). Immunologically active autoantigens: the role of toll-like receptors in the development of chronic inflammatory disease. Annu. Rev. Immunol. *25:419-41.*, 419-441.

Matarrese,P., Fusco,O., Tinari,N., Natoli,C., Liu,F.T., Semeraro,M.L., Malorni,W., and Iacobelli,S. (2000). Galectin-3 overexpression protects from apoptosis by improving cell adhesion properties. Int. J Cancer. *85*, 545-554.

Matarrese,P., Tinari,A., Mormone,E., Bianco,G.A., Toscano,M.A., Ascione,B., Rabinovich,G.A., and Malorni,W. (2005). Galectin-1 sensitizes resting human T lymphocytes to Fas (CD95)-mediated cell death via mitochondrial hyperpolarization, budding, and fission. J Biol Chem. *280*, 6969-6985.

Matsumoto,R., Matsumoto,H., Seki,M., Hata,M., Asano,Y., Kanegasaki,S., Stevens,R.L., and Hirashima,M. (1998). Human Ecalectin, a Variant of Human Galectin-9, Is a Novel Eosinophil Chemoattractant Produced by T Lymphocytes. J. Biol. Chem. *273*, 16976-16984.

Matsumoto,S., Muller-Ladner,U., Gay,R.E., Nishioka,K., and Gay,S. (1996). Ultrastructural demonstration of apoptosis, Fas and Bcl-2 expression of rheumatoid synovial fibroblasts. J. Rheumatol. *23*, 1345-1352.

Matsuura,A., Tsukada,J., Mizobe,T., Higashi,T., Mouri,F., Tanikawa,R., Yamauchi,A., Hirashima,M., and Tanaka,Y. (2009). Intracellular galectin-9 activates inflammatory cytokines in monocytes. Genes Cells. *14*, 511-521.

Mauri,C. and Ehrenstein,M.R. (2007). Cells of the synovium in rheumatoid arthritis. B cells. Arthritis Res. Ther. *9*, 205.

Maurice,M.M., Lankester,A.C., Bezemer,A.C., Geertsma,M.F., Tak,P.P., Breedveld,F.C., van Lier,R.A., and Verweij,C.L. (1997). Defective TCR-mediated signaling in synovial T cells in rheumatoid arthritis. J. Immunol. *159*, 2973-2978.

McInnes,I.B., Leung,B.P., and Liew,F.Y. (2000). Cell-cell interactions in synovitis. Interactions between T lymphocytes and synovial cells. Arthritis Res. *2*, 374-378.

McInnes,I.B. and Schett,G. (2007). Cytokines in the pathogenesis of rheumatoid arthritis. Nat Rev Immunol *7*, 429-442.

Menon,R.P. and Hughes,R.C. (1999). Determinants in the N-terminal domains of galectin-3 for secretion by a novel pathway circumventing the endoplasmic reticulum-Golgi complex. Eur. J Biochem. *264*, 569-576.

Mey,A., Leffler,H., Hmama,Z., Normier,G., and Revillard,J.P. (1996). The animal lectin galectin-3 interacts with bacterial lipopolysaccharides via two independent sites. J Immunol *156*, 1572-1577.

Meyer,L.H., Franssen,L., and Pap,T. (2006). The role of mesenchymal cells in the pathophysiology of inflammatory arthritis. Best. Pract. Res. Clin. Rheumatol. *20*, 969-981.

Miagkov,A.V., Kovalenko,D.V., Brown,C.E., Didsbury,J.R., Cogswell,J.P., Stimpson,S.A., Baldwin,A.S., and Makarov,S.S. (1998). NF-kappaB activation provides the potential link between inflammation and hyperplasia in the arthritic joint. Proc. Natl. Acad. Sci. U. S. A. *95*, 13859-13864.

Miceli,M.C., Moran,M., Chung,C.D., Patel,V.P., Low,T., and Zinnanti,W. (2001). Co-stimulation and counter-stimulation: lipid raft clustering controls TCR signaling and functional outcomes. Semin. Immunol. *13*, 115-128.

Mills,C.D., Kincaid,K., Alt,J.M., Heilman,M.J., and Hill,A.M. (2000). M-1/M-2 Macrophages and the Th1/Th2 Paradigm. J Immunol *164*, 6166-6173.

Min,D.J., Cho,M.L., Lee,S.H., Min,S.Y., Kim,W.U., Min,J.K., Park,S.H., Cho,C.S., and Kim,H.Y. (2004). Augmented production of chemokines by the interaction of type II collagen-reactive T cells with rheumatoid synovial fibroblasts. Arthritis Rheum. *50*, 1146-1155.

Miossec,P. (2004). An update on the cytokine network in rheumatoid arthritis. Curr. Opin. Rheumatol. *16*, 218-222.

Miossec,P. (2003). Interleukin-17 in rheumatoid arthritis: if T cells were to contribute to inflammation and destruction through synergy. Arthritis Rheum. *48*, 594-601.

Miossec,P., Naviliat,M., Dupuy,d.A., Sany,J., and Banchereau,J. (1990). Low levels of interleukin-4 and high levels of transforming growth factor beta in rheumatoid synovitis. Arthritis Rheum. *33*, 1180-1187.

Miranda,T.B. and Jones,P.A. (2007). DNA methylation: the nuts and bolts of repression. J Cell Physiol. *213*, 384-390.

Miyazawa,K., Mori,A., and Okudaira,H. (1998a). Regulation of interleukin-1beta-induced interleukin-6 gene expression in human fibroblast-like synoviocytes by glucocorticoids. J Biochem. *124*, 1130-1137.

Miyazawa,K., Mori,A., Yamamoto,K., and Okudaira,H. (1998b). Constitutive transcription of the human interleukin-6 gene by rheumatoid synoviocytes: spontaneous activation of NF-kappaB and CBF1. Am. J Pathol. *152*, 793-803.

Moisan,S., Demers,M., Mercier,J., Magnaldo,T., Potworowski,E.F., and St Pierre,Y. (2003). Upregulation of galectin-7 in murine lymphoma cells is associated with progression toward an aggressive phenotype. Leukemia. *17*, 751-759.

Moiseeva,E.P., Williams,B., Goodall,A.H., and Samani,N.J. (2003). Galectin-1 interacts with beta-1 subunit of integrin. Biochem. Biophys. Res. Commun. *310*, 1010-1016.

Mulherin,D., Fitzgerald,O., and Bresnihan,B. (1996). Synovial tissue macrophage populations and articular damage in rheumatoid arthritis. Arthritis Rheum. *39*, 115-124.

Muller-Ladner,U., Kriegsmann,J., Franklin,B.N., Matsumoto,S., Geiler,T., Gay,R.E., and Gay,S. (1996). Synovial fibroblasts of patients with rheumatoid arthritis attach to and invade normal human cartilage when engrafted into SCID mice. Am. J. Pathol. *149*, 1607-1615.

Muller-Ladner,U. and Gay,S. (2006). The role of fibroblast-like synoviocytes in rheumatoid arthritis. In Rheumatoid arthritis, G.S.Firestein, G.S.Panayi, and F.A.Wollheim, eds. Oxford Univesity Press), pp. 106-121.

Naka,E.L., Ponciano,V.C., Cenedeze,M.A., Pacheco-Silva,A., and Saraiva CΓmara,N.O. (2009). Detection of the Tim-3 ligand, galectin-9, inside the allograft during a rejection episode. International Immunopharmacology *9*, 658-662.

Nakahara,S., Hogan,V., Inohara,H., and Raz,A. (2006). Importin-mediated nuclear translocation of galectin-3. J Biol. Chem. *281*, 39649-39659.

Nakano,K., Okada,Y., Saito,K., and Tanaka,Y. (2004). Induction of RANKL expression and osteoclast maturation by the binding of fibroblast growth factor 2 to heparan sulfate proteoglycan on rheumatoid synovial fibroblasts. Arthritis Rheum. *50*, 2450-2458.

Nangia-Makker,P., Nakahara,S., Hogan,V., and Raz,A. (2007). Galectin-3 in apoptosis, a novel therapeutic target. J. Bioenerg. Biomembr. ..

Nangia-Makker,P., Honjo,Y., Sarvis,R., Akahani,S., Hogan,V., Pienta,K.J., and Raz,A. (2000). Galectin-3 Induces Endothelial Cell Morphogenesis and Angiogenesis. Am J Pathol *156*, 899-909.

Nanki,T., Nagasaka,K., Hayashida,K., Saita,Y., and Miyasaka,N. (2001). Chemokines Regulate IL-6 and IL-8 Production by Fibroblast-Like Synoviocytes from Patients with Rheumatoid Arthritis. J Immunol *167*, 5381-5385.

Neidhart,M., Hauser,N., Paulsson,M., DiCesare,P.E., Michel,B.A., and Hauselmann,H.J. (1997). Small fragments of cartilage oligomeric matrix protein in synovial fluid and serum as markers for cartilage degradation. Rheumatology *36*, 1151-1160.

Neidhart,M., Zaucke,F., von Knoch,R., Jungel,A., Michel,B.A., Gay,R.E., and Gay,S. (2005). Galectin-3 is induced in rheumatoid arthritis synovial fibroblasts after adhesion to cartilage oligomeric matrix protein. Ann. Rheum. Dis. *64*, 419-424.

Nickel,W. (2003). The mystery of nonclassical protein secretion. A current view on cargo proteins and potential export routes. Eur. J. Biochem. *270*, 2109-2119.

Nielen,M.M., van Schaardenburg,D., Reesink,H.W., van de Stadt,R.J., van der Horst-Bruinsma IE, de Koning,M.H., Habibuw,M.R., Vandenbroucke,J.P., and Dijkmans,B.A. (2004). Specific autoantibodies precede the symptoms of rheumatoid arthritis: a study of serial measurements in blood donors. Arthritis Rheum. *50*, 380-386.

Nieminen,J., St Pierre,C., and Sato,S. (2005). Galectin-3 interacts with naive and primed neutrophils, inducing innate immune responses. J Leukoc Biol *78*, 1127-1135.

Nile,C.J., Read,R.C., Akil,M., Duff,G.W., and Wilson,A.G. (2008). Methylation status of a single CpG site in the IL6 promoter is related to IL6 messenger RNA levels and rheumatoid arthritis. Arthritis Rheum. *58*, 2686-2693.

Nishi,N., Itoh,A., Fujiyama,A., Yoshida,N., Araya,S., Hirashima,M., Shoji,H., and Nakamura,T. (2005). Development of highly stable galectins: truncation of the linker peptide confers protease-resistance on tandem-repeat type galectins. FEBS Lett. *579*, 2058-2064.

Nishi,N., Itoh,A., Shoji,H., Miyanaka,H., and Nakamura,T. (2006). Galectin-8 and galectin-9 are novel substrates for thrombin. Glycobiology *16*, 15C-20.

Nishi,N., Shoji,H., Seki,M., Itoh,A., Miyanaka,H., Yuube,K., Hirashima,M., and Nakamura,T. (2003). Galectin-8 modulates neutrophil function via interaction with integrin {alpha}M. Glycobiology *13*, 755-763.

Nishimoto,N. (2006). Interleukin-6 in rheumatoid arthritis. Curr. Opin. Rheumatol. *18*, 277-281.

Noso,N., Sticherling,M., Bartels,J., Mallet,A.I., Christophers,E., and Schroder,J.M. (1996). Identification of an N-terminally truncated form of the chemokine RANTES and granulocyte-macrophage colony-stimulating factor as major eosinophil attractants released by cytokine-stimulated dermal fibroblasts. J Immunol *156*, 1946-1953.

Numasaki,M., Tomioka,Y., Takahashi,H., and Sasaki,H. (2004). IL-17 and IL-17F modulate GM-CSF production by lung microvascular endothelial cells stimulated with IL-1beta and/or TNF-alpha. Immunol Lett. *95*, 175-184.

Ochieng,J., Green,B., Evans,S., James,O., and Warfield,P. (1998a). Modulation of the biological functions of galectin-3 by matrix metalloproteinases. Biochim. Biophys. Acta. *1379*, 97-106.

Ochieng,J., Leite-Browning,M.L., and Warfield,P. (1998b). Regulation of cellular adhesion to extracellular matrix proteins by galectin-3. Biochem. Biophys. Res. Commun. *246*, 788-791.

Ochieng,J., Warfield,P., Green-Jarvis,B., and Fentie,I. (1999). Galectin-3 regulates the adhesive interaction between breast carcinoma cells and elastin. J Cell Biochem. *75*, 505-514.

Offner,H., Celnik,B., Bringman,T.S., Casentini-Borocz,D., Nedwin,G.E., and Vandenbark,A.A. (1990). Recombinant human beta-galactoside binding lectin suppresses clinical and histological signs of experimental autoimmune encephalomyelitis. J. Neuroimmunol. *28*, 177-184.

Ohannesian,D.W., Lotan,D., Thomas,P., Jessup,J.M., Fukuda,M., Gabius,H.J., and Lotan,R. (1995). Carcinoembryonic antigen and other glycoconjugates act as ligands for galectin-3 in human colon carcinoma cells. Cancer Res. *55*, 2191-2199.

Ohashi,K., Burkart,V., Flohe,S., and Kolb,H. (2000). Cutting Edge: Heat Shock Protein 60 Is a Putative Endogenous Ligand of the Toll-Like Receptor-4 Complex. J Immunol *164*, 558-561.

Ohshima,S., Kuchen,S., Seemayer,C.A., Kyburz,D., Hirt,A., Klinzing,S., Michel,B.A., Gay,R.E., Liu,F.T., Gay,S., and Neidhart,M. (2003). Galectin 3 and its binding protein in rheumatoid arthritis. Arthritis Rheum. *48*, 2788-2795.

Ohtsubo,K. and Marth,J.D. (2006). Glycosylation in cellular mechanisms of health and disease. Cell. *126*, 855-867.

Okudaira,T., Hirashima,M., Ishikawa,C., Makishi,S., Tomita,M., Matsuda,T., Kawakami,H., Taira,N., Ohshiro,K., Masuda,M., Takasu,N., and Mori,N. (2007). A modified version of galectin-9 suppresses cell growth and induces apoptosis of human T-cell leukemia virus type I-infected T-cell lines. Int. J Cancer. *120*, 2251-2261.

Openo,K.P., Kadrofske,M.M., Patterson,R.J., and Wang,J.L. (2000). Galectin-3 expression and subcellular localization in senescent human fibroblasts. Exp. Cell Res. *255*, 278-290.

Ospelt,C., Brentano,F., Rengel,Y., Stanczyk,J., Kolling,C., Tak,P.P., Gay,R.E., Gay,S., and Kyburz,D. (2008). Overexpression of toll-like receptors 3 and 4 in synovial tissue from patients with early rheumatoid arthritis: toll-like receptor expression in early and longstanding arthritis. Arthritis Rheum. *58*, 3684-3692.

Ospelt,C., Kyburz,D., Pierer,M., Seibl,R., Kurowska,M., Distler,O., Neidhart,M., Muller-Ladner,U., Pap,T., Gay,R.E., and Gay,S. (2004a). Toll-like receptors in rheumatoid arthritis joint destruction mediated by two distinct pathways. Ann. Rheum. Dis. *63 Suppl 2:ii90-ii91.*, ii90-ii91.

Ospelt,C., Neidhart,M., Gay,R.E., and Gay,S. (2004b). Synovial activation in rheumatoid arthritis. Front Biosci. *9:2323-34.*, 2323-2334.

Ottonello,L., Cutolo,M., Frumento,G., Arduino,N., Bertolotto,M., Mancini,M., Sottofattori,E., and Dallegri,F. (2002). Synovial fluid from patients with rheumatoid arthritis inhibits neutrophil apoptosis: role of adenosine and proinflammatory cytokines. Rheumatology. (Oxford). *41*, 1249-1260.

Ozaki,K., Inoue,K., Sato,H., Iida,A., Ohnishi,Y., Sekine,A., Sato,H., Odashiro,K., Nobuyoshi,M., Hori,M., Nakamura,Y., and Tanaka,T. (2004). Functional variation in LGALS2 confers risk of myocardial infarction and regulates lymphotoxin-[alpha] secretion in vitro. Nature *429*, 72-75.

Ozeki,Y., Matsui,T., Yamamoto,Y., Funahashi,M., Hamako,J., and Titani,K. (1995). Tissue fibronectin is an endogenous ligand for galectin-1. Glycobiology. *5*, 255-261.

Pace,K.E., Hahn,H.P., Pang,M., Nguyen,J.T., and Baum,L.G. (2000). CD7 delivers a pro-apoptotic signal during galectin-1-induced T cell death. J Immunol. *165*, 2331-2334.

Pace,K.E., Lee,C., Stewart,P.L., and Baum,L.G. (1999). Restricted receptor segregation into membrane microdomains occurs on human T cells during apoptosis induced by galectin-1. J Immunol. *163*, 3801-3811.

Page,G., Lebecque,S., and Miossec,P. (2002). Anatomic Localization of Immature and Mature Dendritic Cells in an Ectopic Lymphoid Organ: Correlation with Selective Chemokine Expression in Rheumatoid Synovium. J Immunol *168*, 5333-5341.

Paleolog,E. (2002). Angiogenesis in rheumatoid arthritis. Arthritis Res *4*, S81-S90.

Pap,T., Franz,J.K., Hummel,K.M., Jeisy,E., Gay,R., and Gay,S. (2000a). Activation of synovial fibroblasts in rheumatoid arthritis: lack of Expression of the tumour suppressor PTEN at sites of invasive growth and destruction. Arthritis Res. *2*, 59-64.

Pap,T., Gay,R.E., and Gay,S. (2006). Mechanisms of fibroblast-mediated joint destruction. In Rheumatoid arthritis, G.S.Firestein, G.S.Panayi, and F.A.Wollheim, eds. Oxford Univeristy Press), pp. 199-212.

Pap,T., Muller-Ladner,U., Gay,R.E., and Gay,S. (2000b). Fibroblast biology. Role of synovial fibroblasts in the pathogenesis of rheumatoid arthritis. Arthritis Res. *2*, 361-367.

Pap,T., Nawrath,M., Heinrich,J., Bosse,M., Baier,A., Hummel,K.M., Petrow,P., Kuchen,S., Michel,B.A., Gay,R.E., Muller-Ladner,U., Moelling,K., and Gay,S. (2004). Cooperation of Ras- and c-Myc-dependent pathways in regulating the growth and invasiveness of synovial fibroblasts in rheumatoid arthritis. Arthritis Rheum. *50*, 2794-2802.

Pap,T., Shigeyama,Y., Kuchen,S., Fernihough,J.K., Simmen,B., Gay,R.E., Billingham,M., and Gay,S. (2000c). Differential expression pattern of membrane-type matrix metalloproteinases in rheumatoid arthritis. Arthritis Rheum. *43*, 1226-1232.

Papaspyridonos,M., McNeill,E., de Bono,J.P., Smith,A., Burnand,K.G., Channon,K.M., and Greaves,D.R. (2008). Galectin-3 Is an Amplifier of Inflammation in Atherosclerotic Plaque Progression Through Macrophage Activation And Monocyte Chemoattraction. Arterioscler Thromb Vasc Biol *28*, 433-440.

Park,J.S., Gamboni-Robertson,F., He,Q., Svetkauskaite,D., Kim,J.Y., Strassheim,D., Sohn,J.W., Yamada,S., Maruyama,I., Banerjee,A., Ishizaka,A., and Abraham,E. (2006). High mobility group box 1 protein interacts with multiple Toll-like receptors. Am J Physiol Cell Physiol. *290*, C917-C924.

Parsonage,G., Falciani,F., Burman,A., Filer,A., Ross,E., Bofill,M., Martin,S., Salmon,M., and Buckley,C.D. (2003). Global gene expression profiles in fibroblasts from synovial, skin and lymphoid tissue reveals distinct cytokine and chemokine expression patterns. Thromb. Haemost. *90*, 688-697.

Parsonage,G., Filer,A., Bik,M., Hardie,D., Lax,S., Howlett,K., Church,L.D., Raza,K., Wong,S.H., Trebilcock,E., Scheel-Toellner,D., Salmon,M., Lord,J.M., and Buckley,C.D. (2008). Prolonged, granulocyte-macrophage colony-stimulating factor-dependent, neutrophil survival following rheumatoid synovial fibroblast activation by IL-17 and TNFalpha. Arthritis Res. Ther. *10*, R47.

Parsonage,G., Filer,A.D., Haworth,O., Nash,G.B., Rainger,G.E., Salmon,M., and Buckley,C.D. (2005). A stromal address code defined by fibroblasts. Trends Immunol. *26*, 150-156.

Patnaik,S.K., Potvin,B., Carlsson,S., Sturm,D., Leffler,H., and Stanley,P. (2006). Complex N-glycans are the major ligands for galectin-1, -3, and -8 on Chinese hamster ovary cells. Glycobiology *16*, 305-317.

Paz,A., Haklai,R., Elad-Sfadia,G., Ballan,E., and Kloog,Y. (2001). Galectin-1 binds oncogenic H-Ras to mediate Ras membrane anchorage and cell transformation. Oncogene. *20*, 7486-7493.

Pedersen,G., Andresen,L., Matthiessen,M.W., Rask-Madsen,J., and Brynskov,J. (2005). Expression of Toll-like receptor 9 and response to bacterial CpG oligodeoxynucleotides in human intestinal epithelium. Clin. Exp. Immunol. *141*, 298-306.

Pelletier,J.P., Martel-Pelletier,J., and Abramson,S.B. (2001). Osteoarthritis, an inflammatory disease: potential implication for the selection of new therapeutic targets. Arthritis Rheum. *44*, 1237-1247.

Pellme,S., Morgelin,M., Tapper,H., Mellqvist,U.H., Dahlgren,C., and Karlsson,A. (2006). Localization of human neutrophil interleukin-8 (CXCL-8) to organelle(s) distinct from the classical granules and secretory vesicles. J Leukoc Biol. *79*, 564-573.

Perillo,N.L., Uittenbogaart,C.H., Nguyen,J.T., and Baum,L.G. (1997). Galectin-1, an Endogenous Lectin Produced by Thymic Epithelial Cells, Induces Apoptosis of Human Thymocytes. J. Exp. Med. *185*, 1851-1858.

Perlman,H., Pagliari,L.J., and Volin,M.V. (2001). Regulation of apoptosis and cell cycle activity in rheumatoid arthritis. Curr. Mol. Med. *1*, 597-608.

Pettit,A.R., MacDonald,K.P., O'Sullivan,B., and Thomas,R. (2000). Differentiated dendritic cells expressing nuclear RelB are predominantly located in rheumatoid synovial tissue perivascular mononuclear cell aggregates. Arthritis Rheum. *43*, 791-800.

Pierer,M., Rethage,J., Seibl,R., Lauener,R., Brentano,F., Wagner,U., Hantzschel,H., Michel,B.A., Gay,R.E., Gay,S., and Kyburz,D. (2004). Chemokine secretion of rheumatoid arthritis synovial fibroblasts stimulated by Toll-like receptor 2 ligands. J. Immunol. *172*, 1256-1265.

Pilling,D., Akbar,A.N., Girdlestone,J., Orteu,C.H., Borthwick,N.J., Amft,N., Scheel-Toellner,D., Buckley,C.D., and Salmon,M. (1999). Interferon-beta mediates stromal cell rescue of T cells from apoptosis. Eur. J Immunol. *29*, 1041-1050.

Platanias,L.C. (2003). The p38 mitogen-activated protein kinase pathway and its role in interferon signaling. Pharmacol. Ther. *98*, 129-142.

Potocnik,A.J., Kinne,R., Menninger,H., Zacher,J., Emmrich,F., and Kroczek,R.A. (1990). Expression of activation antigens on T cells in rheumatoid arthritis patients. Scand. J. Immunol. *31*, 213-224.

Pundt,N., Peters,M.A., Wunrau,C., Strietholt,S., Fehrmann,C., Neugebauer,K., Seyfert,C., van,V.F., Pap,T., and Meinecke,I. (2009). Susceptibility of rheumatoid arthritis synovial fibroblasts to FasL- and TRAIL-induced apoptosis is cell cycle-dependent. Arthritis Res. Ther. *11*, R16.

Ra,H.J. and Parks,W.C. (2007). Control of matrix metalloproteinase catalytic activity. Matrix Biol. *26*, 587-596.

Rabinovich,G.A., Baum,L.G., Tinari,N., Paganelli,R., Natoli,C., Liu,F.T., and Iacobelli,S. (2002). Galectins and their ligands: amplifiers, silencers or tuners of the inflammatory response? Trends Immunol. *23*, 313-320.

Rabinovich,G.A., Sotomayor,C.E., Riera,C.M., Bianco,I., and Correa,S.G. (2000). Evidence of a role for galectin-1 in acute inflammation. Eur. J. Immunol. *30*, 1331-1339.

Rabinovich,G.A. and Toscano,M.A. (2009). Turning 'sweet' on immunity: galectin-glycan interactions in immune tolerance and inflammation. Nat. Rev. Immunol. *9*, 338-352.

Rabinovich,G.A., Toscano,M.A., Jackson,S.S., and Vasta,G.R. (2007). Functions of cell surface galectin-glycoprotein lattices. Curr. Opin. Struct. Biol. *17*, 513-520.

Rabinovich,G.A., Daly,G., Dreja,H., Tailor,H., Riera,C.M., Hirabayashi,J., and Chernajovsky,Y. (1999). Recombinant Galectin-1 and Its Genetic Delivery Suppress Collagen-induced Arthritis via T Cell Apoptosis. J. Exp. Med. *190*, 385-398.

Radstake,T.R., Roelofs,M.F., Jenniskens,Y.M., Oppers-Walgreen,B., van Riel,P.L., Barrera,P., Joosten,L.A., and van den Berg,W.B. (2004). Expression of toll-like receptors 2 and 4 in rheumatoid synovial tissue and regulation by proinflammatory cytokines interleukin-12 and interleukin-18 via interferon-gamma. Arthritis Rheum. *50*, 3856-3865.

Rashid,T. and Ebringer,A. (2007). Rheumatoid arthritis is linked to Proteus--the evidence. Clin. Rheumatol. *26*, 1036-1043.

Rathanaswami,P., Hachicha,M., Sadick,M., Schall,T.J., and McColl,S.R. (1993). Expression of the cytokine RANTES in human rheumatoid synovial fibroblasts.

Differential regulation of RANTES and interleukin-8 genes by inflammatory cytokines. J. Biol. Chem. *268*, 5834-5839.

Raza,K., Falciani,F., Curnow,S.J., Ross,E.J., Lee,C.Y., Akbar,A.N., Lord,J.M., Gordon,C., Buckley,C.D., and Salmon,M. (2005a). Early rheumatoid arthritis is characterized by a distinct and transient synovial fluid cytokine profile of T cell and stromal cell origin. Arthritis Res. Ther. *7*, R784-R795.

Raza K, Breese M, Nightingale P, Kumar K, Potter T, Carruthers DM, Situnayake D, Gordon C, Buckley CD, Salmon M, Kitas GD. (2005b) Predictive value of antibodies to cyclic citrullinated peptide in patients with very early inflammatory arthritis. J Rheumatol. 32(2):231-8

Raza,K., Scheel-Toellner,D., Lee,C.Y., Pilling,D., Curnow,S.J., Falciani,F., Trevino,V., Kumar,K., Assi,L.K., Lord,J.M., Gordon,C., Buckley,C.D., and Salmon,M. (2006). Synovial fluid leukocyte apoptosis is inhibited in patients with very early rheumatoid arthritis. Arthritis Res. Ther. *8*, R120.

Rechreche,H., Mallo,G.V., Montalto,G., Dagorn,J.C., and Iovanna,J.L. (1997). Cloning and expression of the mRNA of human galectin-4, an S-type lectin down-regulated in colorectal cancer. Eur. J Biochem. *248*, 225-230.

Rengel,Y., Ospelt,C., and Gay,S. (2007). Proteinases in the joint: clinical relevance of proteinases in joint destruction. Arthritis Research & Therapy *9*, 221.

Rinaldi,N., Schwarz-Eywill,M., Weis,D., Leppelmann-Jansen,P., Lukoschek,M., Keilholz,U., and Barth,T.F. (1997). Increased expression of integrins on fibroblast-like synoviocytes from rheumatoid arthritis in vitro correlates with enhanced binding to extracellular matrix proteins. Ann. Rheum. Dis. *56*, 45-51.

Rinn,J.L., Bondre,C., Gladstone,H.B., Brown,P.O., and Chang,H.Y. (2006). Anatomic demarcation by positional variation in fibroblast gene expression programs. PLoS. Genet. *2*, e119.

Rissoan,M.C., Van,K.C., Chomarat,P., Galibert,L., Durand,I., Thivolet-Bejui,F., Miossec,P., and Banchereau,J. (1996). The functional CD40 antigen of fibroblasts may contribute to the proliferation of rheumatoid synovium. Clin. Exp. Immunol. *106*, 481-490.

Ritchlin,C. (2000). Fibroblast biology. Effector signals released by the synovial fibroblast in arthritis. Arthritis Res. *2*, 356-360.

Ritchlin,C., Dwyer,E., Bucala,R., and Winchester,R. (1994). Sustained and distinctive patterns of gene activation in synovial fibroblasts and whole synovial tissue obtained from inflammatory synovitis. Scand. J. Immunol. *40*, 292-298.

Rivollier,A., Mazzorana,M., Tebib,J., Piperno,M., Aitsiselmi,T., Rabourdin-Combe,C., Jurdic,P., and Servet-Delprat,C. (2004). Immature dendritic cell

transdifferentiation into osteoclasts: a novel pathway sustained by the rheumatoid arthritis microenvironment. Blood *104*, 4029-4037.

Roelofs,M.F., Boelens,W.C., Joosten,L.A., bdollahi-Roodsaz,S., Geurts,J., Wunderink,L.U., Schreurs,B.W., van den Berg,W.B., and Radstake,T.R. (2006). Identification of small heat shock protein B8 (HSP22) as a novel TLR4 ligand and potential involvement in the pathogenesis of rheumatoid arthritis. J Immunol. *176*, 7021-7027.

Roman-Blas,J.A. and Jimenez,S.A. (2006). NF-[kappa]B as a potential therapeutic target in osteoarthritis and rheumatoid arthritis. Osteoarthritis and Cartilage *14*, 839-848.

Ross,E.A., Douglas,M.R., Wong,S.H., Ross,E.J., Curnow,S.J., Nash,G.B., Rainger,E., Scheel-Toellner,D., Lord,J.M., Salmon,M., and Buckley,C.D. (2006). Interaction between integrin {alpha}9beta1 and vascular cell adhesion molecule-1 (VCAM-1) inhibits neutrophil apoptosis. Blood *107*, 1178-1183.

Rubinstein,N., Ilarregui,J.M., Toscano,M.A., and Rabinovich,G.A. (2004). The role of galectins in the initiation, amplification and resolution of the inflammatory response. Tissue Antigens. *64*, 1-12.

Ruddy,M.J., Wong,G.C., Liu,X.K., Yamamoto,H., Kasayama,S., Kirkwood,K.L., and Gaffen,S.L. (2004). Functional cooperation between interleukin-17 and tumor necrosis factor-alpha is mediated by CCAAT/enhancer-binding protein family members. J. Biol. Chem. *279*, 2559-2567.

Ruebel,K.H., Jin,L., Qian,X., Scheithauer,B.W., Kovacs,K., Nakamura,N., Zhang,H., Raz,A., and Lloyd,R.V. (2005). Effects of DNA Methylation on Galectin-3 Expression in Pituitary Tumors. Cancer Res *65*, 1136-1140.

Sacre,S.M., Drexler,S.K., Andreakos,E., Feldmann,M., Brennan,F.M., and Foxwell,B.M.J. (2007). Could toll-like receptors provide a missing link in chronic inflammation in rheumatoid arthritis? Lessons from a study on human rheumatoid tissue. Ann Rheum Dis *66*, iii81-iii86.

Saegusa,J., Hsu,D.K., Chen,H.Y., Yu,L., Fermin,A., Fung,M.A., and Liu,F.T. (2009). Galectin-3 Is Critical for the Development of the Allergic Inflammatory Response in a Mouse Model of Atopic Dermatitis. Am J Pathol *174*, 922-931.

Salamone,G., Giordano,M., Trevani,A.S., Gamberale,R., Vermeulen,M., Schettinni,J., and Geffner,J.R. (2001). Promotion of Neutrophil Apoptosis by TNF-{{alpha}}. J Immunol *166*, 3476-3483.

Salmon,M., Scheel-Toellner,D., Huissoon,A.P., Pilling,D., Shamsadeen,N., Hyde,H., D'Angeac,A.D., Bacon,P.A., Emery,P., and Akbar,A.N. (1997). Inhibition of T cell apoptosis in the rheumatoid synovium. J Clin. Invest. *99*, 439-446.

Samuels,J., Krasnokutsky,S., and Abramson,S.B. (2008). Osteoarthritis: a tale of three tissues. Bull. NYU. Hosp. Jt. Dis. *66*, 244-250.

Sanchez-Pernaute,O., Ospelt,C., Neidhart,M., and Gay,S. (2008). Epigenetic clues to rheumatoid arthritis. Journal of Autoimmunity *30*, 12-20.

Sano,H., Hsu,D.K., Apgar,J.R., Yu,L., Sharma,B.B., Kuwabara,I., Izui,S., and Liu,F.T. (2003). Critical role of galectin-3 in phagocytosis by macrophages. J Clin. Invest. *112*, 389-397.

Sano,H., Hsu,D.K., Yu,L., Apgar,J.R., Kuwabara,I., Yamanaka,T., Hirashima,M., and Liu,F.T. (2000). Human Galectin-3 Is a Novel Chemoattractant for Monocytes and Macrophages. J Immunol *165*, 2156-2164.

Santucci,L., Fiorucci,S., Cammilleri,F., Servillo,G., Federici,B., and Morelli,A. (2000). Galectin-1 exerts immunomodulatory and protective effects on concanavalin A-induced hepatitis in mice. Hepatology. *31*, 399-406.

Santucci,L., Fiorucci,S., Rubinstein,N., Mencarelli,A., Palazzetti,B., Federici,B., Rabinovich,G.A., and Morelli,A. (2003). Galectin-1 suppresses experimental colitis in mice. Gastroenterology. *124*, 1381-1394.

Sarkissian,M. and Lafyatis,R. (1999). Integrin engagement regulates proliferation and collagenase expression of rheumatoid synovial fibroblasts. J. Immunol. *162*, 1772-1779.

Sarter,K., Andrθ,S., Kaltner,H., Lensch,M., Schulze,C., Urbonaviciute,V., Schett,G., Herrmann,M., and Gabius,H.J. (2009). Detection and chromatographic removal of lipopolysaccharide in preparations of multifunctional galectins. Biochemical and Biophysical Research Communications *379*, 155-159.

Sato,S. and Nieminen,J. (2004). Seeing strangers or announcing "danger": galectin-3 in two models of innate immunity. Glycoconj. J. *19*, 583-591.

Sato,S., Ouellet,N., Pelletier,I., Simard,M., Rancourt,A., and Bergeron,M.G. (2002). Role of Galectin-3 as an Adhesion Molecule for Neutrophil Extravasation During Streptococcal Pneumonia. J Immunol *168*, 1813-1822.

Saussez,S. and Kiss,R. (2006). Galectin-7. Cell Mol. Life Sci. *63*, 686-697.

Savill,J.S., Henson,P.M., and Haslett,C. (1989). Phagocytosis of aged human neutrophils by macrophages is mediated by a novel "charge-sensitive" recognition mechanism. J Clin. Invest. *84*, 1518-1527.

Scaife,S., Brown,R., Kellie,S., Filer,A., Martin,S., Thomas,A.M.C., Bradfield,P.F., Amft,N., Salmon,M., and Buckley,C.D. (2004). Detection of differentially expressed genes in synovial fibroblasts by restriction fragment differential display. Rheumatology *43*, 1346-1352.

Scapini,P., Lapinet-Vera,J.A., Gasperini,S., Calzetti,F., Bazzoni,F., and Cassatella,M.A. (2000). The neutrophil as a cellular source of chemokines. Immunol. Rev. *177:195-203.*, 195-203.

Scheel-Toellner,D., Wang,K., Henriquez,N.V., Webb,P.R., Craddock,R., Pilling,D., Akbar,A.N., Salmon,M., and Lord,J.M. (2002). Cytokine-mediated inhibition of apoptosis in non-transformed T cells and neutrophils can be dissociated from protein kinase B activation. Eur. J Immunol. *32*, 486-493.

Schett,G., Tohidast-Akrad,M., Smolen,J.S., Schmid,B.J., Steiner,C.W., Bitzan,P., Zenz,P., Redlich,K., Xu,Q., and Steiner,G. (2000). Activation, differential localization, and regulation of the stress-activated protein kinases, extracellular signal-regulated kinase, c-JUN N-terminal kinase, and p38 mitogen-activated protein kinase, in synovial tissue and cells in rheumatoid arthritis. Arthritis Rheum. *43*, 2501-2512.

Schiller,J., Arnhold,J., Sonntag,K., and Arnold,K. (1996). NMR studies on human, pathologically changed synovial fluids: role of hypochlorous acid. Magn Reson. Med. *35*, 848-853.

Schindler,C., Levy,D.E., and Decker,T. (2007). JAK-STAT Signaling: From Interferons to Cytokines. J. Biol. Chem. *282*, 20059-20063.

Schnare,M., Barton,G.M., Holt,A.C., Takeda,K., Akira,S., and Medzhitov,R. (2001). Toll-like receptors control activation of adaptive immune responses. Nat. Immunol. *2*, 947-950.

Schroder,A.E., Greiner,A., Seyfert,C., and Berek,C. (1996). Differentiation of B cells in the nonlymphoid tissue of the synovial membrane of patients with rheumatoid arthritis. Proc. Natl. Acad. Sci. U. S. A. *93*, 221-225.

Schroder,A.K., Uciechowski,P., Fleischer,D., and Rink,L. (2006). Crosslinking of CD66B on peripheral blood neutrophils mediates the release of interleukin-8 from intracellular storage. Hum. Immunol. *67*, 676-682.

Schurigt,U., Pfirschke,C., Irmler,I.M., Huckel,M., Gajda,M., Janik,T., Baumgrass,R., Bernhagen,J., and Brauer,R. (2008). Interactions of T helper cells with fibroblast-like synoviocytes: up-regulation of matrix metalloproteinases by macrophage migration inhibitory factor from both Th1 and Th2 cells. Arthritis Rheum. *58*, 3030-3040.

Schwartz,M.A. (1997). Integrins, oncogenes, and anchorage independence. J. Cell Biol. *139*, 575-578.

Seelenmeyer,C., Wegehingel,S., Tews,I., Kunzler,M., Aebi,M., and Nickel,W. (2005). Cell surface counter receptors are essential components of the unconventional export machinery of galectin-1. J. Cell Biol. *171*, 373-381.

Seibl,R., Birchler,T., Loeliger,S., Hossle,J.P., Gay,R.E., Saurenmann,T., Michel,B.A., Seger,R.A., Gay,S., and Lauener,R.P. (2003). Expression and regulation of Toll-like receptor 2 in rheumatoid arthritis synovium. Am. J Pathol. *162*, 1221-1227.

Seki,M., Oomizu,S., Sakata,K.M., Sakata,A., Arikawa,T., Watanabe,K., Ito,K., Takeshita,K., Niki,T., Saita,N., Nishi,N., Yamauchi,A., Katoh,S., Matsukawa,A.,

Kuchroo,V., and Hirashima,M. (2008). Galectin-9 suppresses the generation of Th17, promotes the induction of regulatory T cells, and regulates experimental autoimmune arthritis. Clin. Immunol. *127*, 78-88.

Seki,M., Sakata,K.M., Oomizu,S., Arikawa,T., Sakata,A., Ueno,M., Nobumoto,A., Niki,T., Saita,N., Ito,K., Dai,S.Y., Katoh,S., Nishi,N., Tsukano,M., Ishikawa,K., Yamauchi,A., Kuchroo,V., and Hirashima,M. (2007). Beneficial effect of galectin 9 on rheumatoid arthritis by induction of apoptosis of synovial fibroblasts. Arthritis Rheum. *56*, 3968-3976.

Seki,T., Selby,J., Haupl,T., and Winchester,R. (1998). Use of differential subtraction method to identify genes that characterize the phenotype of cultured rheumatoid arthritis synoviocytes. Arthritis Rheum. *41*, 1356-1364.

Shimura,T., Takenaka,Y., Tsutsumi,S., Hogan,V., Kikuchi,A., and Raz,A. (2004). Galectin-3, a Novel Binding Partner of {beta}-Catenin. Cancer Res *64*, 6363-6367.

Siala,M., Gdoura,R., Fourati,H., Rihl,M., Jaulhac,B., Younes,M., Sibilia,J., Baklouti,S., Bargaoui,N., Sellami,S., Sghir,A., and Hammami,A. (2009). Broad-range PCR, cloning and sequencing of the full 16S rRNA gene for detection of bacterial DNA in synovial fluid samples of Tunisian patients with reactive and undifferentiated arthritis. Arthritis Res Ther. *11*, R102.

Silverman,G.J. (2006). The roles of B cells in rheumatoid arthritis. In Rheumatoid arthrits, G.S.Firestein, G.S.Panayi, and F.A.Wollheim, eds. Oxford University Press), pp. 123-134.

Simon,A.K., Seipelt,E., and Sieper,J. (1994). Divergent T-cell cytokine patterns in inflammatory arthritis. Proc. Natl. Acad. Sci. U. S. A. *91*, 8562-8566.

Smith,M.D., Triantafillou,S., Parker,A., Youssef,P.P., and Coleman,M. (1997a). Synovial membrane inflammation and cytokine production in patients with early osteoarthritis. J. Rheumatol. *24*, 365-371.

Smith,R.S., Smith,T.J., Blieden,T.M., and Phipps,R.P. (1997b). Fibroblasts as sentinel cells. Synthesis of chemokines and regulation of inflammation. Am. J. Pathol. *151*, 317-322.

Smolen,J.S., Aletaha,D., Koeller,M., Weisman,M.H., and Emery,P. (2007). New therapies for treatment of rheumatoid arthritis. The Lancet *370*, 1861-1874.

Sone,H., Sakauchi,M., Takahashi,A., Suzuki,H., Inoue,N., Iida,K., Shimano,H., Toyoshima,H., Kawakami,Y., Okuda,Y., Matsuo,K., and Yamada,N. (2001). Elevated levels of vascular endothelial growth factor in the sera of patients with rheumatoid arthritis correlation with disease activity. Life Sci. *69*, 1861-1869.

Southey,A.K., O'Connor,C.M., and Fitzgerald,M.X. (1994). The effect of fibroblast conditioned medium on neutrophil survival and activation. Biochem. Soc. Trans. *22*, 52S.

Spitzenberger,F., Graessler,J., and Schroeder,H.E. (2001). Molecular and functional characterization of galectin 9 mRNA isoforms in porcine and human cells and tissues. Biochimie *83*, 851-862.

Stamp,L.K., Cleland,L.G., and James,M.J. (2004). Upregulation of synoviocyte COX-2 through interactions with T lymphocytes: role of interleukin 17 and tumor necrosis factor-alpha. J. Rheumatol. *31*, 1246-1254.

Sticherling,M., Kupper,M., Koltrowitz,F., Bornscheuer,E., Kulke,R., Klinger,M., Wilhelm,D., Kameyoshi,Y., Christophers,E., and Schroder,J.M. (1995). Detection of the chemokine RANTES in cytokine-stimulated human dermal fibroblasts. J Invest Dermatol. *105*, 585-591.

Stillman,B.N., Hsu,D.K., Pang,M., Brewer,C.F., Johnson,P., Liu,F.T., and Baum,L.G. (2006). Galectin-3 and Galectin-1 Bind Distinct Cell Surface Glycoprotein Receptors to Induce T Cell Death. J Immunol *176*, 778-789.

Stowell,S.R., Karmakar,S., Stowell,C.J., Dias-Baruffi,M., McEver,R.P., and Cummings,R.D. (2007). Human galectin-1, -2, and -4 induce surface exposure of phosphatidylserine in activated human neutrophils but not in activated T cells. Blood. *109*, 219-227.

Strietholt,S., Maurer,B., Peters,M., Pap,T., and Gay,S. (2008). Epigenetic modifications in rheumatoid arthritis. Arthritis Research & Therapy *10*, 219.

Sturm,A., Lensch,M., Andre,S., Kaltner,H., Wiedenmann,B., Rosewicz,S., Dignass,A.U., and Gabius,H.J. (2004). Human Galectin-2: Novel Inducer of T Cell Apoptosis with Distinct Profile of Caspase Activation. J Immunol *173*, 3825-3837.

Suzuki,M.M. and Bird,A. (2008). DNA methylation landscapes: provocative insights from epigenomics. Nat. Rev. Genet. *9*, 465-476.

Swarte,V.V., Mebius,R.E., Joziasse,D.H., Van den Eijnden,D.H., and Kraal,G. (1998). Lymphocyte triggering via L-selectin leads to enhanced galectin-3-mediated binding to dendritic cells. Eur. J Immunol. *28*, 2864-2871.

Szekanecz,Z. and Koch,A.E. (2007). Macrophages and their products in rheumatoid arthritis. Curr. Opin. Rheumatol. *19*, 289-295.

Szekanecz,Z. and Koch,A.E. (2000). Cell-cell interactions in synovitis. Endothelial cells and immune cell migration. Arthritis Res. *2*, 368-373.

Tak,P.P. (2006). Examination of the synovium and synovial fluid. In Rheumatoid arthrits, G.S.Firestein, G.S.Panayi, and F.A.Wollheim, eds. Oxford University Press), pp. 229-241.

Tak,P.P., Smeets,T.J., Daha,M.R., Kluin,P.M., Meijers,K.A., Brand,R., Meinders,A.E., and Breedveld,F.C. (1997). Analysis of the synovial cell infiltrate in early rheumatoid synovial tissue in relation to local disease activity. Arthritis Rheum. *40*, 217-225.

Takemura,S., Braun,A., Crowson,C., Kurtin,P.J., Cofield,R.H., O'Fallon,W.M., Goronzy,J.J., and Weyand,C.M. (2001). Lymphoid Neogenesis in Rheumatoid Synovitis. J Immunol *167*, 1072-1080.

Takenaka,Y., Fukumori,T., and Raz,A. (2004a). Galectin-3 and metastasis. Glycoconj. J. *19*, 543-549.

Takenaka,Y., Fukumori,T., Yoshii,T., Oka,N., Inohara,H., Kim,H.R.C., Bresalier,R.S., and Raz,A. (2004b). Nuclear Export of Phosphorylated Galectin-3 Regulates Its Antiapoptotic Activity in Response to Chemotherapeutic Drugs. Mol. Cell. Biol. *24*, 4395-4406.

Tamura,M., Gu,J., Matsumoto,K., Aota,S., Parsons,R., and Yamada,K.M. (1998). Inhibition of cell migration, spreading, and focal adhesions by tumor suppressor PTEN. Science. *280*, 1614-1617.

Termeer,C., Benedix,F., Sleeman,J., Fieber,C., Voith,U., Ahrens,T., Miyake,K., Freudenberg,M., Galanos,C., and Simon,J.C. (2002). Oligosaccharides of Hyaluronan activate dendritic cells via toll-like receptor 4. J Exp. Med. *195*, 99-111.

Than,N.G., Sumegi,B., Than,G.N., Berente,Z., and Bohn,H. (1999). Isolation and sequence analysis of a cDNA encoding human placental tissue protein 13 (PP13), a new lysophospholipase, homologue of human eosinophil Charcot-Leyden Crystal protein. Placenta. *20*, 703-710.

Toh,M.L. and Miossec,P. (2007). The role of T cells in rheumatoid arthritis: new subsets and new targets. Curr. Opin. Rheumatol. *19*, 284-288.

Toivanen,P. (2006). Microbes in the pathogenesis of rheumatoid arthrits. In Rheumatoid arthritis, G.S.Firestein, G.S.Panayi, and F.A.Wollheim, eds. Oxford University Press), pp. 32-40.

Tolboom,T.C., van der Helm-Van Mil AH, Nelissen,R.G., Breedveld,F.C., Toes,R.E., and Huizinga,T.W. (2005). Invasiveness of fibroblast-like synoviocytes is an individual patient characteristic associated with the rate of joint destruction in patients with rheumatoid arthritis. Arthritis Rheum. *52*, 1999-2002.

Toscano,M.A., Bianco,G.A., Ilarregui,J.M., Croci,D.O., Correale,J., Hernandez,J.D., Zwirner,N.W., Poirier,F., Riley,E.M., Baum,L.G., and Rabinovich,G.A. (2007). Differential glycosylation of TH1, TH2 and TH-17 effector cells selectively regulates susceptibility to cell death. Nat. Immunol. *8*, 825-834.

Tran,C.N., Davis,M.J., Tesmer,L.A., Endres,J.L., Motyl,C.D., Smuda,C., Somers,E.C., Chung,K.C., Urquhart,A.G., Lundy,S.K., Kovats,S., and Fox,D.A. (2007). Presentation of arthritogenic peptide to antigen-specific T cells by fibroblast-like synoviocytes. Arthritis Rheum. *56*, 1497-1506.

Tran,C.N., Lundy,S.K., and Fox,D.A. (2005). Synovial biology and T cells in rheumatoid arthritis. Pathophysiology. *12*, 183-189.

Trebilcock, E. Fibroblast Responses to Galectins. 2006. University of Birmingham.
Ref Type: Thesis/Dissertation

Tribulatti,M.V., Cattaneo,V., Hellman,U., Mucci,J., and Campetella,O. (2009). Galectin-8 provides costimulatory and proliferative signals to T lymphocytes. J. Leukoc. Biol.

Tsark,E.C., Wang,W., Teng,Y.C., Arkfeld,D., Dodge,G.R., and Kovats,S. (2002). Differential MHC class II-mediated presentation of rheumatoid arthritis autoantigens by human dendritic cells and macrophages. J. Immunol. *169*, 6625-6633.

Tsuboi,Y., Abe,H., Nakagawa,R., Oomizu,S., Watanabe,K., Nishi,N., Nakamura,T., Yamauchi,A., and Hirashima,M. (2007). Galectin-9 protects mice from the Shwartzman reaction by attracting prostaglandin E2-producing polymorphonuclear leukocytes. Clin. Immunol. *124*, 221-233.

Tureci,O., Schmitt,H., Fadle,N., Pfreundschuh,M., and Sahin,U. (1997). Molecular Definition of a Novel Human Galectin Which Is Immunogenic in Patients with Hodgkin's Disease. J. Biol. Chem. *272*, 6416-6422.

van Amelsfort,J.M., Jacobs,K.M., Bijlsma,J.W., Lafeber,F.P., and Taams,L.S. (2004). CD4(+)CD25(+) regulatory T cells in rheumatoid arthritis: differences in the presence, phenotype, and function between peripheral blood and synovial fluid. Arthritis Rheum. *50*, 2775-2785.

van de Weyer,P.S., Muehlfeit,M., Klose,C., Bonventre,J.V., Walz,G., and Kuehn,E.W. (2006). A highly conserved tyrosine of Tim-3 is phosphorylated upon stimulation by its ligand galectin-9. Biochem. Biophys. Res. Commun. *351*, 571-576.

van den Berg,J.M., Weyer,S., Weening,J.J., Roos,D., and Kuijpers,T.W. (2001). Divergent effects of tumor necrosis factor {alpha} on apoptosis of human neutrophils. J Leukoc Biol *69*, 467-473.

van den Brule,F.A., Califice,S., and Castronovo,V. (2004). Expression of galectins in cancer: a critical review. Glycoconj. J. *19*, 537-542.

Vas,V., Fajka-Boja,R., Ion,G., Dudics,V., Monostori,E., and Uher,F. (2005). Biphasic Effect of Recombinant Galectin-1 on the Growth and Death of Early Hematopoietic Cells. Stem Cells *23*, 279-287.

Vasta,G.R. (2009). Roles of galectins in infection. Nat. Rev. Microbiol. *7*, 424-438.

Velvart,M. and Fehr,K. (1987). Degradation in vivo of articular cartilage in rheumatoid arthritis and juvenile chronic arthritis by cathepsin G and elastase from polymorphonuclear leukocytes. Rheumatol. Int. *7*, 195-202.

Vereecken,P., Zouaoui,B.K., Debray,C., Awada,A., Legssyer,I., Sales,F., Petein,M., Vanhaeverbeek,M., Ghanem,G., and Heenen,M. (2006). High serum galectin-3 in advanced melanoma: preliminary results. Clin. Exp. Dermatol. *31*, 105-109.

Vespa,G.N.R., Lewis,L.A., Kozak,K.R., Moran,M., Nguyen,J.T., Baum,L.G., and Miceli,M.C. (1999). Galectin-1 Specifically Modulates TCR Signals to Enhance TCR Apoptosis but Inhibit IL-2 Production and Proliferation. J Immunol *162*, 799-806.

Wada,J., Ota,K., Kumar,A., Wallner,E.I., and Kanwar,Y.S. (1997). Developmental regulation, expression, and apoptotic potential of galectin-9, a beta-galactoside binding lectin. J Clin. Invest. *99*, 2452-2461.

Wagner,U.G., Koetz,K., Weyand,C.M., and Goronzy,J.J. (1998). Perturbation of the T cell repertoire in rheumatoid arthritis. Proc. Natl. Acad. Sci. U. S. A. *95*, 14447-14452.

Walmsley,S.R., Cowburn,A.S., Sobolewski,A., Murray,J., Farahi,N., Sabroe,I., and Chilvers,E.R. (2004). Characterization of the survival effect of tumour necrosis factor-alpha in human neutrophils. Biochem. Soc. Trans. *32*, 456-460.

Walmsley,S.R., Print,C., Farahi,N., Peyssonnaux,C., Johnson,R.S., Cramer,T., Sobolewski,A., Condliffe,A.M., Cowburn,A.S., Johnson,N., and Chilvers,E.R. (2005). Hypoxia-induced neutrophil survival is mediated by HIF-1alpha-dependent NF-kappaB activity. J. Exp. Med. *201*, 105-115.

Wang,F., He,W., Zhou,H., Yuan,J., Wu,K., Xu,L., and Chen,Z.K. (2007). The Tim-3 ligand galectin-9 negatively regulates CD8+ alloreactive T cell and prolongs survival of skin graft. Cellular Immunology *250*, 68-74.

Wang,J.L., Gray,R.M., Haudek,K.C., and Patterson,R.J. (2004). Nucleocytoplasmic lectins. Biochim. Biophys. Acta. *1673*, 75-93.

Wang,K., Scheel-Toellner,D., Wong,S.H., Craddock,R., Caamano,J., Akbar,A.N., Salmon,M., and Lord,J.M. (2003). Inhibition of Neutrophil Apoptosis by Type 1 IFN Depends on Cross-Talk Between Phosphoinositol 3-Kinase, Protein Kinase C-{delta}, and NF-{kappa}B Signaling Pathways. J Immunol *171*, 1035-1041.

Wang,L., Inohara,H., Pienta,K.J., and Raz,A. (1995). Galectin-3 Is a Nuclear Matrix Protein Which Binds RNA. Biochemical and Biophysical Research Communications *217*, 292-303.

Watson,R.W., Rotstein,O.D., Nathens,A.B., Parodo,J., and Marshall,J.C. (1997). Neutrophil apoptosis is modulated by endothelial transmigration and adhesion molecule engagement. J Immunol *158*, 945-953.

Wells,V. and Mallucci,L. (1992). Molecular expression of the negative growth factor murine beta-galactoside binding protein (mGBP). Biochim. Biophys. Acta. *1121*, 239-244.

Weyand,C.M. and Goronzy,J.J. (2003). Ectopic germinal center formation in rheumatoid synovitis. Ann. N Y. Acad. Sci. *987:140-9.*, 140-149.

Wilkinson,N.Z., Kingsley,G.H., Jones,H.W., Sieper,J., Braun,J., and Ward,M.E. (1999). The detection of DNA from a range of bacterial species in the joints of patients with a variety of arthritides using a nested, broad-range polymerase chain reaction. Rheumatology *38*, 260-266.

Williams,A.S., Richards,P.J., Thomas,E., Carty,S., Nowell,M.A., Goodfellow,R.M., Dent,C.M., Williams,B.D., Jones,S.A., and Topley,N. (2007). Interferon-gamma protects against the development of structural damage in experimental arthritis by regulating polymorphonuclear neutrophil influx into diseased joints. Arthritis Rheum. *56*, 2244-2254.

Wipke,B.T. and Allen,P.M. (2001). Essential Role of Neutrophils in the Initiation and Progression of a Murine Model of Rheumatoid Arthritis. J Immunol *167*, 1601-1608.

Wong,S.H. and Lord,J.M. (2004). Factors underlying chronic inflammation in rheumatoid arthritis. Arch. Immunol Ther. Exp. (Warsz.). *52*, 379-388.

Yamada,H., Nakashima,Y., Okazaki,K., Mawatari,T., Fukushi,J.i., Kaibara,N., Hori,A., Iwamoto,Y., and Yoshikai,Y. (2007). Th1 but not Th17 cells predominate in the joints of patients with rheumatoid arthritis. Ann Rheum Dis ard.

Yamada,T., Fujieda,S., Yanagi,S., Yamamura,H., Inatome,R., Yamamoto,H., Igawa,H., and Saito,H. (2001). IL-1 Induced Chemokine Production Through the Association of Syk with TNF Receptor-Associated Factor-6 in Nasal Fibroblast Lines. J Immunol *167*, 283-288.

Yamamura,Y., Gupta,R., Morita,Y., He,X., Pai,R., Endres,J., Freiberg,A., Chung,K., and Fox,D.A. (2001). Effector function of resting T cells: activation of synovial fibroblasts. J. Immunol. *166*, 2270-2275.

Yang,Q.S., Ying,K., Yuan,H.L., Chen,J.Z., Meng,X.F., Wang,Z., Xie,Y., and Mao,Y.M. (2002). Cloning and expression of a novel human galectin cDNA, predominantly expressed in placenta(1). Biochim. Biophys. Acta. *1574*, 407-411.

Yang,R.Y., Hsu,D.K., and Liu,F.T. (1996). Expression of galectin-3 modulates T-cell growth and apoptosis. Proc. Natl. Acad. Sci. U. S. A. *93*, 6737-6742.

Yang,R.Y., Hsu,D.K., Yu,L., Ni,J., and Liu,F.T. (2001). Cell cycle regulation by galectin-12, a new member of the galectin superfamily. J. Biol. Chem. *276*, 20252-20260.

Yang,R.Y. and Liu,F.T. (2003). Galectins in cell growth and apoptosis. Cell Mol. Life Sci. *60*, 267-276.

Yoshii,T., Fukumori,T., Honjo,Y., Inohara,H., Kim,H.R., and Raz,A. (2002). Galectin-3 phosphorylation is required for its anti-apoptotic function and cell cycle arrest. J. Biol. Chem. *277*, 6852-6857.

Young,A. and Koduri,G. (2007). Extra-articular manifestations and complications of rheumatoid arthritis. Best Practice & Research Clinical Rheumatology *21*, 907-927.

Zamzami,N., Marchetti,P., Castedo,M., Decaudin,D., Macho,A., Hirsch,T., Susin,S.A., Petit,P.X., Mignotte,B., and Kroemer,G. (1995). Sequential reduction of mitochondrial transmembrane potential and generation of reactive oxygen species in early programmed cell death. J. Exp. Med. *182*, 367-377.

Zare,F., Bokarewa,M., Nenonen,N., Bergstrom,T., Alexopoulou,L., Flavell,R.A., and Tarkowski,A. (2004). Arthritogenic Properties of Double-Stranded (Viral) RNA. J Immunol *172*, 5656-5663.

Zhang,X., Nakajima,T., Goronzy,J.J., and Weyand,C.M. (2005). Tissue trafficking patterns of effector memory CD4+ T cells in rheumatoid arthritis. Arthritis Rheum. *52*, 3839-3849.

Zhu,C., Anderson,A.C., Schubart,A., Xiong,H., Imitola,J., Khoury,S.J., Zheng,X.X., Strom,T.B., and Kuchroo,V.K. (2005). The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. Nat. Immunol. *6*, 1245-1252.

Zhuo,Y., Chammas,R., and Bellis,S.L. (2008). Sialylation of beta1 integrins blocks cell adhesion to galectin-3 and protects cells against galectin-3-induced apoptosis. J. Biol. Chem.

Zimmermann,T., Kunisch,E., Pfeiffer,R., Hirth,A., Stahl,H.D., Sack,U., Laube,A., Liesaus,E., Roth,A., Palombo-Kinne,E., Emmrich,F., and Kinne,R.W. (2001). Isolation and characterization of rheumatoid arthritis synovial fibroblasts from primary culture--primary culture cells markedly differ from fourth-passage cells. Arthritis Res. *3*, 72-76.

Ziolkowska,M., Koc,A., Luszczykiewicz,G., Ksiezopolska-Pietrzak,K., Klimczak,E., Chwalinska-Sadowska,H., and Maslinski,W. (2000). High Levels of IL-17 in Rheumatoid Arthritis Patients: IL-15 Triggers In Vitro IL-17 Production Via Cyclosporin A-Sensitive Mechanism. J Immunol *164*, 2832-2838.

Zuberi,R.I., Frigeri,L.G., and Liu,F.T. (1994). Activation of rat basophilic leukemia cells by epsilon BP, an IgE-binding endogenous lectin. Cell Immunol. *156*, 1-12.

Zuberi,R.I., Hsu,D.K., Kalayci,O., Chen,H.Y., Sheldon,H.K., Yu,L., Apgar,J.R., Kawakami,T., Lilly,C.M., and Liu,F.T. (2004). Critical role for galectin-3 in airway inflammation and bronchial hyperresponsiveness in a murine model of asthma. Am. J. Pathol. *165*, 2045-2053.

Zuniga,E., Rabinovich,G.A., Iglesias,M.M., and Gruppi,A. (2001). Regulated expression of galectin-1 during B-cell activation and implications for T-cell apoptosis. J Leukoc Biol *70*, 73-79.

Zvaifler,N.J. (2006).
Clinical aspects of rheumatoid arthrits. In Rheumatoid arthrits, G.S.Firestein, G.S.Panayi, and F.A.Wollheim, eds. Oxford University Press), pp. 245-257.