Role Of Pathogenic Auto-Antibodies And Innate Immunity Mediators In K/BxN Murine Model For Rheumatoid Arthritis

Dissertation

Zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften

an der Universität Konstanz

Fakultät für Biologie

vorgelegt von

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Konstanz, im Dezember 2002

Tag der mündlichen Prüfung: 12. März 2003

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I will instruct thee and teach thee in the way which thou shalt go.
I will guide thee with mine eye.
Psalms 32:8

Dedicated to Appa, Amma & Babu.

My special thanks to Dr. Harald Illges, for giving an opportunity to carry out this work, for financial support from his research grants, for his guidance and helpful discussions.

My special thanks to Prof. Dr. Rolf Knippers for being my doctoral father and for his valuable guidance during my PhD studies.

My sincere thanks to Prof. Dr. Marcus Groettrup for his support and for accepting to be a referee for my PhD thesis and oral examinations.

My sincere thanks to Prof. Dr. W. Welte for accepting to be my examiner for oral examinations.

I am greatly indebted to Dr. Cornelia Kolb for her help, especially in establishing the monoclonal antibodies.

My sincere thanks to Prof. Michael Glocker for letting me, to make use of the mass spectrometry facility at the Proteome center, University Rostock.

Thanks to Elvira Jeisy for the technical assistance in histology.

My sincere thanks to Uli beck, Elisabeth Naidoo, Elisabeth von Seydlitz, Alex Titz, and Brigitte Shanze for their valuable assistance in my work.

Finally I thank all my lab colleagues at University Konstanz, Jorg Schwab, Attef Allam, Subhasis Mohanty, Madhan Masilamani, Lawrence Rajendran, Daniella Kasshahn, Narendiran Rajasekaran and at BITG, Melanie Hoefer, Vera Klett, Eva Maria, and Annette Aichem for their kind help and co-operation.

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Ab	antibody
Ag	antigen
AT	ankle thickness
BSA	bovine serum albumin
cDNA	complementary DNA
CI	clinical index
CIA	collagen induced arthritis
CO_2	carbondioxide
CR1	complement receptor 1
CR2	complement receptor 2
CVF	cobra venom factor
DEPC	diethylene pyrocarbonate
DMSO	dimethylsulfoxide
DNA	deoxy ribonucleic acid
DTT	Dithiothreitol
E. coli	escherichia coli
e.g.	for example
ECM	extra cellular matrix
EDTA	Ethylene diaminetetra acetic acid
ELISA	enzyme linked immunosorbant assay
EtBr	ethidium bromide
FACS	flourescent activated cell scan
FcγR	fc gamma receptor
FCS	fetal calf serum
FITC	flourescien isothicyanate
FPLC	fast protein liquid chromatography
GAPDH	glyceraldehyde phosphate dehydrogenase
GPI	glucose–6-phosphate isomerase
GRO	growth related oncogene
GST	glutathione –s-transferase
h	hour
HAT	hypoxanthine aminopterin thymidine
HLA	human leucocyte antigen
HPLC	high performance liquid chromatography
HRP	horse radish peroxidase
i.p	intra peritoneal
i.v	intra venous
IC	immune complex
ICAM-1	intra cellular adhesion molecule-1
Ig	immunoglobulin
IL	interleukin
IMDEM	iscoves dulbecco´s modified medium
IPTG	isopropyl-beta-D-thiogalactopyranoside
KDa	kilo daltons
M, mM	molar, millimolar
mA	milli amphere
MALDI-	matrix assisted laser desorption ionisation
MCP	monocyte chemoattractant protein
MeOH	methanol
MHC	major histocompatibility complex
MIP-2	macrophage inflammatory protein
MMP	matrix metallo protease
MP	milk powder
NO	nitric oxide
NTP	nucleotide tri phosphate
^oC	degree celsius
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PAR	protease activated receptor
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PEG	polyethyleneglycol
PMN	polymorphonuclear monocytes
RA	rheumatoid arthritis
RNA	ribonucleic acid
RPM	rotations per minute
RT	room temperature
SDS	sodium dodecyl sulfate
SEM	standard error mean
SFM	serum free medium
SLE	systemic lupus erythematosus
TAE	tris acetate edta
TBE	tris borate edta
TBS	tris buffered saline
TBST	tris buffered saline tween
TCR	t cell recptor
TE	tris edta
TFA	triflouroacetic acid
TNFα	tumour necrosis factor α
TNFR	tumour necrosis factor receptor
ul	micro liter

UV	ultra violet	v/v , w/v	volume/volume, weight/volume
V	volt	VCAM	vascular cell adhesion molecule

1.0. Introduction

1.1. Scope for Rheumatoid Arthritis research.

Rheumatoid Arthritis (RA) is chronic progressive, systemic inflammatory disorder affecting the synovial joints. It affects 1 to 2% of population, with prevalence increasing with age, and can reach upto 5% in women over age 55. If left untreated it leads to joint destruction responsible for the deformity and disability seen in the disease. The resulting morbidity and mortality has a substantial socio-economic impact on the society.

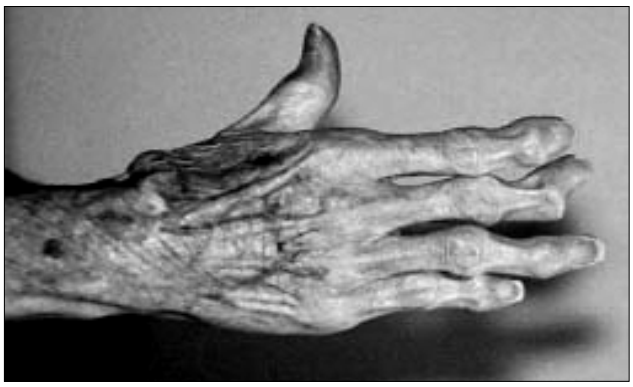

Figure 1. Affected limb of a patient with Rheumatoid Arthritis.

The etiology of RA is still not very clear. Even a basic question whether it is primarily an autoimmune disease, or an inflammatory response/ to infection, is yet to be resolved. Genetic predisposition has been implicated, while failure to demonstrate Mendelian inheritance means multiple genetic factors are probably involved. In humans the MHC class II HLA-DR4 allele is shown to be associated with development and severity of RA. Controversy exists also about the role of the effector leukocyte populations in RA pathogenesis. The dominating paradigm in the field until recently was that antigen-specific T cells in the joint incites the inflammatory cascade by triggering macrophages and synoviocytes. But key observations such as paucity of T cell and its derived cytokines in inflamed joints disapproved such views. Recent studies of new animal models have shifted this importance to B-cell secreted autoantibodies and innate immunity mediators (Benoist and Mathis 2000). The current concept being that while T and B cells are important in initiation of RA the pathogenicity is mostly mediated by autoantibodies and innate immune mediators. The role of inflammatory cytokines such as IL-1 and TNFα are well known and are current targets in therapy. The emerging key role of autoantibodies and innate immunity mediators has renewed hopes for discovery of new targets for RA treatment.

1.2. KBN spontaneous mice model for Rheumatoid Arthritis-*a paradigm shift from T cell centric to an autoantibodies centric view of RA pathogenesis.*

Murine RA models like collagen or pristane induced arthritis models have been of tremendous use in RA research. These animals models have been parallels to understand human RA, yet are more simple to elucidate RA mediators, for hypothesis and drug testing. Generally these models were studied with a notion that T cell, through interaction with antigen, is the primary cell responsible for initiating the disease as well as for driving the chronic inflammatory process by activating macrophages and fibroblasts. However a very recent model of RA the spontaneous RA K/BxN murine model (Kouskoff, Korganow et al. 1996) has changed this thought. RA in these mice develops spontaneously in the offspring of the αβ-TCR-transgenic (specific for RNAse peptide residues 41–61) KRN mice crossed with the NOD diabetes mice strain harboring the MHC II A^{g7} allele (figure 3). RA development in K/BxN offsprings is visible at about 3 weeks of age, characterized by rapid onset of joint inflammation and cartilage destruction. The pathology is similar in several aspects to human RA, with symmetrical involvement of peripheral joints, pannus formation, synovial hyperplasia, bone and cartilage destruction, with subsequent anarchic remodeling of the joints.

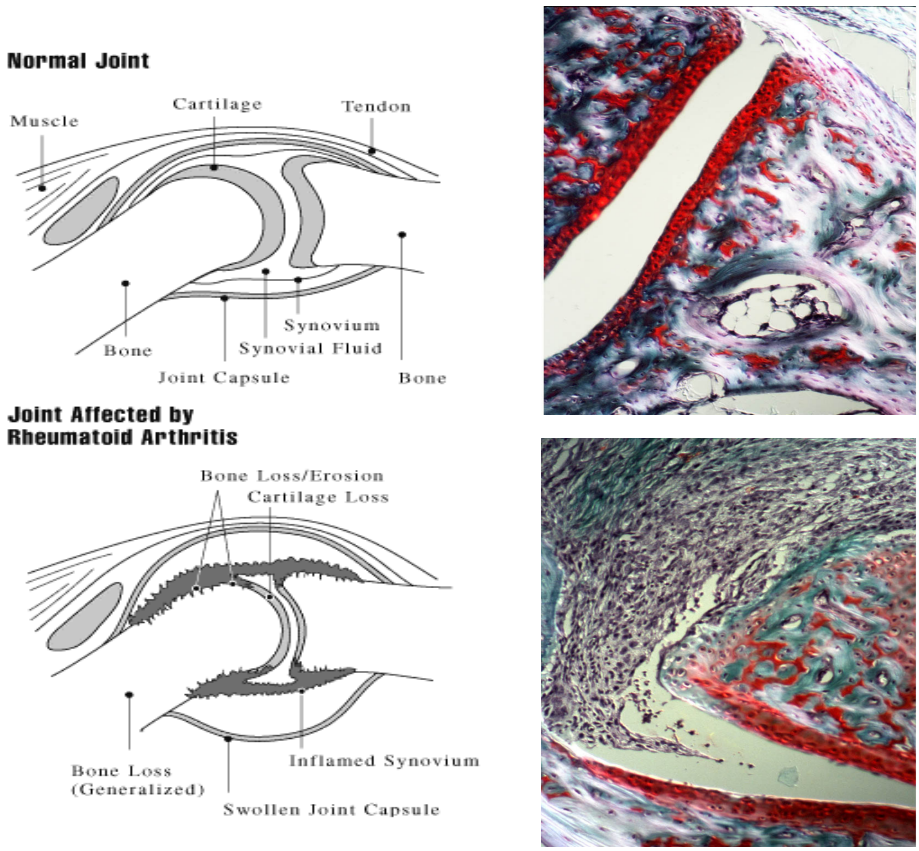

Figure 2: Illustration of joint tissue in normal health and RA with parallels of ankle joint histology of normal and Rheumatoid Arthritis affected joint in mice.

In the K/BxN mice RA model, initiation of RA is dependent on KRN T cells. But unexpectedly the B-cell immunoglobulins were found to be the arthritogenic effectors as the disease could be transferred to naïve mice and also in mice lacking T and B cells by injection K/BxN mice immunoglobulins (Korganow, Ji et al. 1999).

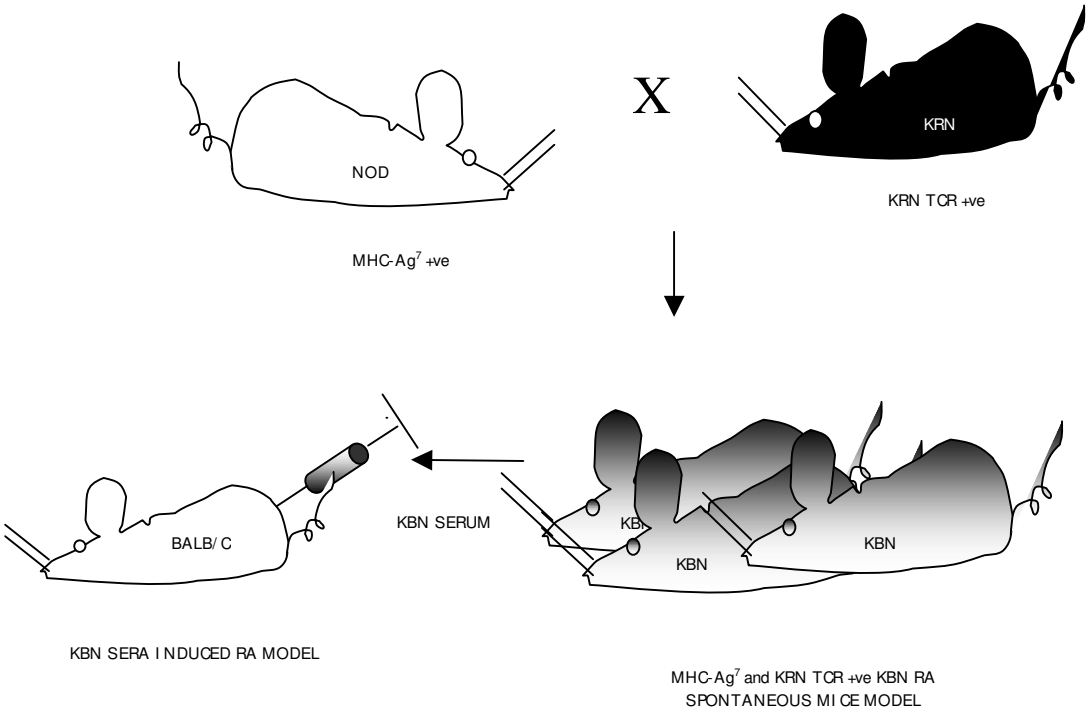

Figure 3: Illustration showing generation of the K/BxN spontaneous RA mice model.

Subsequent studies showed that the disease depends on the KRN T cell recognition of a ubiquitous glycolytic enzyme, glucose-6-phosphate isomerase (GPI), on I-A^{g7} MHC-II molecule. The arthritogenic sera of sick K/BxN mice also recognized a 60KDa band in cell lysates, corresponding to ubiquitous glyolytic enzyme glucose-6-phosphate isomerase (GPI) (Matsumoto, Staub et al. 1999). Transfer of, as little as 100 μl of K/BxN serum or GPI affinity purified antibodies could induce arthritis in naive recipient mice and in mice lacking lymphocytes. The T-cell epitopes (GPI residues 282-294) on GPI have been reported (Basu, Horvath et al. 2000) but the B-cell epitopes of arthritogenic GPI specific antibody have not yet been elucidated. The joint tissue specificity of this very interesting RA model is also yet to be clearly explained. Recently it was shown that GPI deposited on ankle joint surfaces of sick as well normal mice, could probably target GPI antibodies to the joints (Matsumoto, Maccioni et al. 2002). Also recently (Schaller, Burton et al. 2001) a study, showed that 64% of humans with RA had increased concentrations of anti-GPI antibodies in serum and synovial fluid.

1.3. Note on auto-antigen in KBN mice model-*an ubiquitous antigen and much more.*

Several proteins like collagen, gp39, proteoglycans, RA33, p205, and citrullinated proteins are implicated as autoantigens in RA. glucose-6-phosphate isomerase has been the most recent in this list. An interesting point to be noted is the multiple functions of GPI, GPI as a homodimer, in cytosol functions as glycoltic enzyme catalyzing the reversible isomerization of glucose 6-phosphate to fructose 6-phosphate, whereas as a monomeric phosphorlyated form it is secreted from the cytoplasm and can be detected at low concentration (0.24-0.54 *u*g/ml) in serum. Several interesting extracellular functions have been attributed to the monomeric GPI, like in mineralization of osteoblasts (Zhi, Sommerfeldt et al. 2001), in stimulation of B-cells to secrete immunoglobulins (Gurney, Apatoff et al. 1986), as an angiogenic factor that stimulates endothelial cell motility (Funasaka, Haga et al. 2001) and in survival of spinal and sensory neurons (Gurney, Heinrich et al. 1986). Interestingly also the GPI receptor (gp78) is a seven membrane G-protein coupled receptor, a receptor most often implicated in inflammation, cell activation and motility (Shimizu, Tani et al. 1999). Till date no investigations have been carried out with a view that GPI protein as such could have role in pathogenesis of RA. It can be expected that the tissue specificity in RA depends on a putative joint specific antigen. In fact, immunization of mice with collagen type II a protein found in joints induces pathogenic autoantibodies targeted to murine type II collagen in joints and resulting in arthritis affecting joints(collagen-induced arthritis, CIA) (Holmdahl, Mo et al. 1989). The autoimmune target in KBN RA model is a ubiquitous glycolytic enzyme, GPI (Matsumoto, Staub et al. 1999). The question how a ubiquitous protein leads to joint specific autoimmunity and systemic disease has been one of the most puzzling questions in this model. Also how T cell tolerance is broken for this enzyme is a unresolved question in this RA model. No evidence has been provided for the possibility of modified forms of GPI or mimicry antigens being recognized by KRN T cells or the by GPI autoantibodies till now.

1.4.Innate immunity mediators of inflammation in Rheumatoid Arthritis.

1.4.1. Role of autoantibodies in RA– *a tissue specificity target for innate immune system.*

A important question in RA pathogenesis had been, whether immune complexes represent primary disease pathology mediators or are "innocent bystanders" resulting from tissue damage. But now increasing evidence exists from mice models (Stuart and Dixon 1983; Korganow, Ji et al. 1999) that autoantibodies are principle effectors for the initiation and propagation of joint inflammation in RA. Immune complexes targeted to joints can lead to complement activation and/or directly effect cell activation through Fcγ receptors(Baumann, Kohl et al. 2000; Heyman 2000). The complement cascade so initiated can recruit cell mediators of inflammation by the activating and chemo-attractant property of complement activation products. Post-translational modification on antibodies like lack of galactose on asparagine-linked oligosaccharides on the Ig CH2 domain has been associated with severity of RA(Rademacher, Williams et al. 1994). Also these antibodies have impaired immune complex clearance and are defective in down regulating activated B cells. Most often immune complexes are targeted to the site of inflammation due to the presence of auto-antigen. Hence immunizing mice with joint/cartilage protein like collagen or proteogylcan can induce RA affecting joints. Recent studies from mice models have shown strong parallels between auto-antigens recognized by autoantibodies in human RA and mice. Collagen-II antibodies in RA patients was shown to recognize the very same pathogenic epitopes on collagen-II as in CIA mice model (Burkhardt, Koller et al. 2002). Likewise some RA patients have high titers of GPI autoantibodies reflecting the similarities of the KBN mice model to human RA(Schaller, Burton et al. 2001; Kassahn, Kolb et al. 2002; Schubert, Schmidt et al. 2002). The elucidation of the reason for pathogenicity and epitopes recognized by the GPI antibodies in KBN mice would be of utmost importance in the above context.

1.4.2. Role of complements and complement activation products-*an opsonic handle for complement receptors and more.*

The formation and deposition of soluble immune complexes(IC) results in a variety of autoimmune disease like arthritis, vasculitis and glomerulo-nephritis. Generally initiation of the immune complex mediated tissue injury is mainly attributed to the activation of the classical complement pathway, while bacterial products are known to activate the alternative or lectin complement pathway (figure 4). The deposition of immune complexes allows binding and activation of early complement components 1, 4 and 2 of the classical complement pathway resulting in the formation of a C3-convertase. The classical pathway C3

convertase cleaves C3 leading to C3b-deposition on ICs through covalent bonding. A further product of this proteolytic process is the formation of the C5-convertase, leading finally to the assembly of the membrane attack complex (MAC).

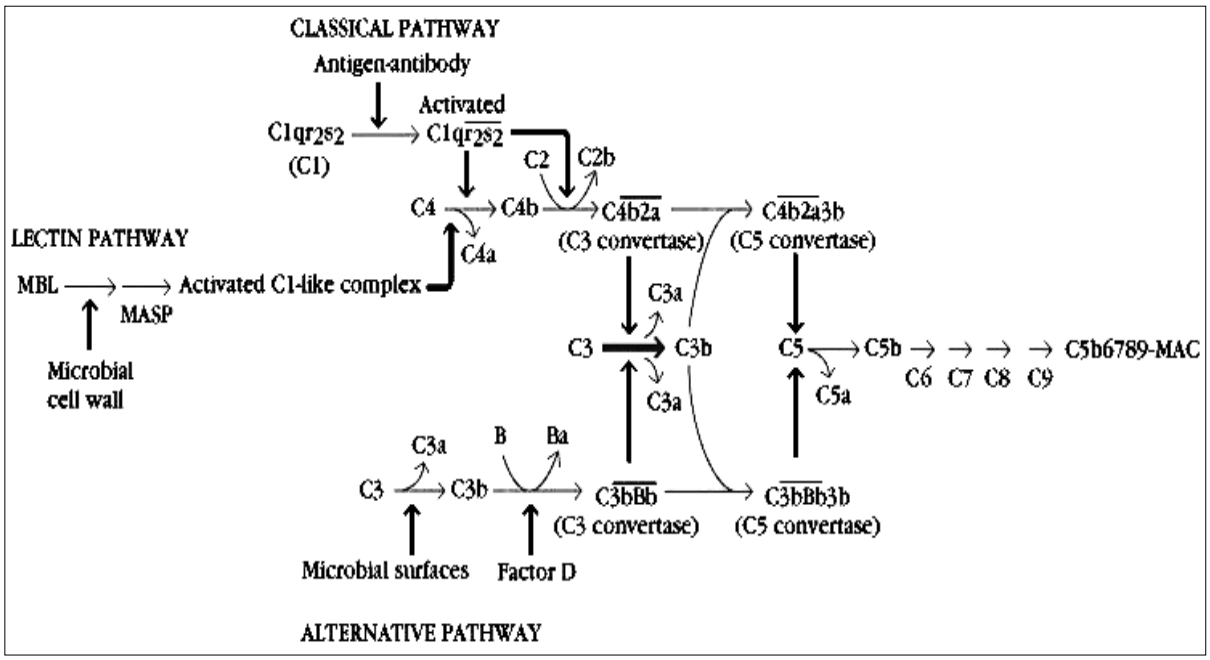

Figure 4: An illustration showing the complement activation pathways. (taken from Kuby, Immunology, 4th Ed).

A common outcome of complement activation is the covalent attachment of the C3 split product, C3b, iC3b and C3d, to the target surface. These products modulate the inflammatory process by providing the opsonic handle recognized by the complement receptors (CR1/CD35, CR2/CD21, and CR3/CD11b-CD18) expressed on leukocytes. The small complement activation byproducts C3a and C5a are potent chemotactic peptides and induce mast cell/neutrophil influx and degranulation leading to tissue injury (Baumann 2001). Rightly so complement C3-deficient mice have reduced or no inflammation due to reduced mast cell degranulation, TNFα production and decreased neutrophil infiltration (Prodeus, Zhou et al. 1997). Also the complement C5 deficient mice have defective inflammatory responses (Wang, Kristan et al. 2000), which strongly implicate the role of complement activation and C5a in activating cell mediators. Also complement proteins are implicated in IC-clearance e.g. a deficiency in the C4 complement component results in a systemic lupus erythematosus (SLE)-like disease in humans (Gatenby 1991).

1.4.3. Role of Complement receptors in RA-*Regulators of inflammation.*

Complement receptors especially CR1(CD35), CR2(CD21) and C5aR(CD88) can have a modulatory role on the outcome and severity of autoimmune inflammatory disease. Complement activation products C3b and C4b can attach covalently to proteins/antibodies, also these and other complement split products (C4b, C4d, iC3b, C3dg and C3d) are ligands for complement receptors CR1 and CR2. The complement receptor CR1 is known to have an important role in the clearance of IC from the circulation, in inhibition of C3 and C5 convertase (decay accelerating activity) and as a receptor for C3b/C4b products (Krych-Goldberg and Atkinson 2001). In SLE, the disposal of IC fails as a consequence of a disease-induced reduction in the number of CR1 on erythrocytes resulting in deposition of IC in glomeruli and small vessels leading to inflammatory reactions (Gatenby 1991). Another clear evidence of protective role for CR1 comes from the reduced disease severity of CIA in mice over expressing soluble CR1 (Dreja, Annenkov et al. 2000). The complement receptor CR2 has a role in inflammation by being a receptor for C3dg-bound immune complexes, in reducing the threshold for B cell activation, involved in germinal center reactions and in the activation of the alternative complement pathway on binding iC3 (Schwendinger, Spruth et al. 1997). Also C5a receptor has been strongly implicated in inflammation (Höpken 1997; Baumann, Kohl et al. 2000). Genetic deletion of C5aR leads to impaired inflammatory responses in mice (Höpken 1997). C5aR on mast cells and chondrocytes are strongly up regulated in RA (Kiener, Baghestanian et al. 1998; Onuma, Masuko-Hongo et al. 2002). The triggering of mast cell de-granulation by the complement activation product C5a links the immune complex mediated complement activation to the innate immunity cellular component in RA inflammation.

1.4.4. Role of Fcγ Receptors in RA-*linking antibodies to effector cells.*

The Fc component of immune complexes can directly interact with the cellular components of innate immune system via Fcγ receptors (FcγRs) and thereby link antibody-antigen complexes to innate immune effector cell activation. FcγRs, belong to the immunoglobulin superfamily that bind IgG. Three classes of leucocyte FcγR have been described (FcγRI, RII, and RIII) (Dearon 1997). Binding of these receptors initiates signaling cascades that can lead to either activation(RI and RIII) or deactivation(RII) of effector cells. The coordinated expression of activating and inhibitory Fcγ receptors has been suggested to drive immune-complex-mediated diseases. In recent studies, the importance of these receptors in inflammation and tissue damage in various autoimmune and inflammatory diseases have been highlighted by

several knockout animal studies. *FcγRIII*$^{-/-}$ mice show a marked reduction of inflammatory cytokines TNFα and IL-1β in inflammatory responses. Cross-linking of FcγRs on monocytes leads to the production of inflammatory cytokines (Kindt, Moore et al. 1993) and chemokines (Marsh, Wewers et al. 1997). *FcγRIII*$^{-/-}$ mice are highly protected from IgG-induced hemolytic anemia, CIA (Stahl, Andren et al. 2002) and show an impaired Arthus reaction (Baumann, Kohl et al. 2000), suggesting a dominant role of FcγRIII (Marsh, Gadek et al. 1995). Also strong correlation exist on FcγRI/III expression on macrophages and the severity of cartilage destruction in human RA (Heller, Gessner et al. 1999; Blom, van Lent et al. 2000).

1.4.5. Role of cellular mediators of inflammation in RA-*The key innate immunity cellular triad-Mast cell, Neutrophil and Macrophage.*

1.4.5.1. Role of Mast cell and mediators- *triggers in inflammation- a link between complement activation to cellular inflammation.*

Mast cells have a key role as `triggers´ of inflammatory responses. Mast cells are strategically located at high density around the blood vessels that are the principal sites of plasma efflux and leukocyte transmigration. Activation of mast cells leads to immediate degranulation and release of stored inflammation mediators such as TNFα, vasoactive mediators like histamine, proteases like tryptase and chemokines like IL-8 (Krishnaswamy, Kelley et al. 2001). Mast cells along with complement activation are implicated in immune-complex induced injury (Baumann 2001); zymosan induced peritoneal inflammation (Kolaczkowska 2001) and in Arthus reaction (Sylvestre and Ravetch 1996). Mast cells are implicated in production of the first wave of TNFα secretion, recruiting neutrophils to sites of inflammation (von Stebut, Metz et al. 2002). Moreover synovial mast cells have been known to up regulate C5aR during inflammation (Kiener, Baghestanian et al. 1998) and activation of mast cells by C5a results in inflammatory cytokine secretion (Woolley and Tetlow 2000). An important vaso-active amine mediator stored in mast cell granules is histamine**.** Histamine has diverse functions including inducing local dilation of small vessels and increase vascular permeability by contraction of endothelial cells. Histamine functions by activating three G coupled histamine receptors H1, H2 and H3 receptors (Del Valle and Gantz 1997; Baroody and Naclerio 2000). Histamine is also a potent inducer of IL-8 from monocytes (Kohka, Nishibori et al. 2000). Also the serine proteases tryptase, plasmin and thrombin released by mast cells are implicated in degradation of matrix macromolecules like fibronectin, fibrinogen, type VI collagen and in activation of cells by cleaving protease activated receptors (PAR) (Akers, Parsons et al. 2000). Mast cells

also serve as functional interface between the neuroendocrine and the immune system as a number of neuropeptides (e.g. substance P, calcitonin gene–related peptide, VIP and the related pituitary adenylate cyclase-activating polypeptide) have been reported to induce mast cell degranulation (Stassen, Hultner et al. 2002).

1.4.5.2. Role of neutrophils- *mediator of acute inflammation.*

Polymorphonuclear leukocytes (PMN) are the most abundant leukocytes, comprising about two-thirds of peripheral blood leukocytes. Upon tissue injury and inflammation circulating PMNs infiltrate rapidly into inflamed tissue. Recent evidence suggests that Neutrophils are recruited by mast cells to sites of inflammation by producing TNFα and MIP-2 (functional murine homologue of human IL-8) (Biedermann, Kneilling et al. 2000; von Stebut, Metz et al. 2002). Neutrophil are also known to be recruited to sites of complement activation by C5a (Höpken 1997). Activated neutrophils produce and release pro-inflammatory mediators, such as IL-1, IL-8, macrophage inflammatory protein (MIP) and proteases (Scapini, Lapinet-Vera et al. 2000). Neutrophils have been clearly implicated in the recruitment of macrophages into sites of inflammation by producing macrophage chemoattractants MIP-1α/β, MCP-1 (von Stebut, Metz et al. 2002) and inactive myeloperoxidase (iMPO) (Lefkowitz and Lefkowitz 2001). Neutrophils are involved in local production of complement C3 at sites of inflammation (Yu, Tsai et al. 1995) leading to a positive feedback loop of neutrophil-complement activation axis. Also the respiratory burst activity of Neutrophils can lead to tissue destruction by reactive nitrogen and oxygen radicals and inducible NO synthase2 (*iNOS2*) or gp91 (*phox*) gene knockout mice are defective in inflammation. Induction of neutropenia leads to complete reduction of inflammation and macrophage infiltration in animal models (Chen 2001; Wipke and Allen 2001). IL8-R (CXCR2) knockout mice are defective for neutrophil migration proving the important role of MIP-2 and other related CXC chemokine (White 1998; Godaly, Hang et al. 2000).

1.4.5.3. Role of Macrophages-*a factory of pro-inflammatory cytokines and mediator of chronic inflammation.*

Macrophages have been described as `factories´ of pro-inflammatory cytokines and hence have key role in inflammation and RA (Kinne, Bräuer et al. 2000). Macrophages have a broad pro-inflammatory, tissue destructive and remodeling capacity both during acute and chronic inflammatory conditions. At sites of tissue destruction macrophages produce high amounts of inflammatory cytokines TNFα, IL1-β, IL-8, prostaglandins and tissue degrading proteases-

stromelysin, collagenase, gelatinase B and leukocyte elastase. FcγR crosslinking on monocytes induces TNFα and IL-1β secretion (Kindt, Moore et al. 1993). Activation through CD40 further enhances their release (Wagner, Stout et al. 1994). These cells are present in high numbers in inflamed tissues especially at the cartilage-pannus interface in RA and correlate with severity of disease. Depletion/inactivation of monocytes/macrophages by drugs or inhibiting macrophage migration to sites of inflammation markedly reduces inflammation and tissue destruction (Ricote, Li et al. 1998; Richards, Williams et al. 1999). Macrophages are also involved in the tissue degrading cascade by activation of fibroblasts/osteoclasts in RA. Synovial monocytes are also known to differentiate into osteoclasts in RA leading to bone damage (Danks, Sabokbar et al. 2002). Activation of macrophages FcγR by immune complexes at sites of inflammation seems to be crucial in the pathogenesis of RA as the abundance of FcγR expression is shown strongly correlated to the extent of cartilage destruction (Heller, Gessner et al. 1999; Blom, van Lent et al. 2000). Activated monocytes express chemokine receptors CCR1, CCR2 and CCR5 and are predominantly known to be recruited to sites of inflammation by local production of chemokines MCP-1, RANTES, MIP-1α/ and IL-8 (Gillitzer and Goebeler 2001). Recent studies have shown that monocytes are recruited to sites of inflammation through CCR2 receptors by MCP-1 and after differentiation into macrophages, up regulation of CCR1 and CCR5 leads to predominant recruitment by MIP-1α (Kaufmann, Salentin et al. 2001). Macrophages are also activated by/ attracted to protease degraded extra-cellular matrix components like fibronectin (White, Livant et al. 2001) and hyaluronan (Horton, Shapiro et al. 1999) at sites of tissue damage during inflammation.

1.5. Role of inflammatory cytokines and receptors in Rheumatoid Arthritis- *The TNFα-IL-1β connection.*

1.5.1 TNFα super-family and TNFα receptors-*key role in initiation of inflammation.*

Tumor Necrosis Factor α(TNFα) is one of the first inflammatory cytokine produced during inflammation and has a pivotal role in the cytokine cascade that results in joint inflammation and destruction in rheumatoid arthritis (RA). The role of TNFα in RA has to do with the up regulation of the key pro-inflammatory cytokine IL-1 and other mediators like cyclooxygenase-1 (COX-1), prostaglandin E2 (PGE2), nitric oxide (NO), adhesion molecules, chemokines, collagenases and apoptosis. In initial stages of inflammation degranulating mast cells release stored TNFα to recruit neutrophils (Chen 2001; Stebut 2002). Inhibition of TNFα production has been shown to reduce inflammation and tissue damage (Shealy,

Wooley et al. 2002). The blockade of TNFα and its receptor TNFR1 and TNFR2 with TNFα antibodies and soluble TNFR has been one of much talked about contemporary therapies for RA (Shanahan and St Clair 2002). Though such a therapy has been successful in reducing joint inflammation in RA about 30% of the patients do not respond to the therapy. Recently studies with *TNFα*$^{-/-}$ mice have shown that RA could proceed in the absence of TNFα (Campbell, O'Donnell et al. 2001) or TNFR (Mori, Iselin et al. 1996). In contrast collagen-II mAb induced RA mice model administration of neutralizing TNFα antibodies protected from RA (Kagari, Doi et al. 2002). These studies question the obligate role/requirement of TNFα in disease pathogenesis. The TNF family member receptor activator of NFκB ligand (RANKL) mediates joint damage as the RANK/RANKL interaction is required for the generation and differentiation of osteoclasts (Theill, Boyle et al. 2002). Deregulated RANK/RANKL expression and pathogenic bone resorption by activated osteoclasts have been implicated in RA. In CIA model blockade of RANKL by osteoprotegerin, a RANKL homologue protects from cartilage and bone damage (Romas, Sims et al. 2002).

1.5.2. Role of Interleukin–1β-*a central cytokine in inflammation and joint damage.*

Interleukin-1β (IL-1β) is a key inflammatory cytokine in RA. Transgenic mice over-expressing IL-1β develop full-blown destructive inflammatory arthritis, while complete protection from RA was seen in absence of IL-1β in mice implicating the dominant role of this cytokine (Joosten, Helsen et al. 1999; Ji, Pettit et al. 2002; Kagari, Doi et al. 2002). IL-1β stimulates production of inflammation mediators PGE2, NO, matrix metallo-proteases (MMPs), and COX-2. IL-1 is also implicated in the migration of neutrophils, lymphocytes, and macrophage to the synovium. IL-1 plays a dominating role in cartilage thinning and destruction by activating osteoclast and enhancing secretion of tissue degrading enzymes (Tatakis 1993). *IL-1*$^{-/-}$ mice were shown to be protected from collagen monoclonal antibody induced RA (Kagari, Doi et al. 2002). Blockade of IL-1β in rheumatoid arthritis patients, by an IL-1 receptor antagonist, suppressed inflammatory symptoms and reduced the rate of progression of joint destruction (Cohen, Hurd et al. 2002).

1.6. Role of chemokines and chemokine receptors in RA-*gateways to inflammation.*

Chemokines are key molecules that activate and direct the migration of different types of leucocytes from the blood stream into sites of infection and inflammation. Chemokines mediate their pro-inflammatory effects by binding to a variety of specific receptors, belonging to the G protein-coupled receptors. The CXC chemokines with ELR motif also have

important roles in angiogenesis (Belperio 2000). Table 1 reviews the major chemokines and their receptors and the cells involved.

Table 1. Chemokines and chemokine receptors.

Receptor	Ligands	Main cell type
CXCR1	IL-8	neutrophils
CXCR2	IL-8, NAP-2, Groα, ENA-78	neutrophils
CXCR3	IP-10, MIG, ITAC	activated T cells (TH1)
CXCR4	SDF-1	naïve T cells, B cells
CXCR5	BCA-1	B cells
CCR1	MCP-3, RANTES, MIP-1α	activated T cells, monocytes, eosinophils,DCs
CCR2	MCP-1,-2,-3,-4,-5	monocytes, macrophages, activated T cells
CCR3	eotaxin, MCP-3,-4; RANTES	eosinophils; basophils, activated T cells (TH2)
CCR4	TARC, MIP-1α, RANTES, MDC	activated T cells (TH2); basophils; platelets
CCR5	MIP-1β, RANTES, MIP-1α	activated T cells, monocyte/macrophages; DCs
CCR6	MIP-3α	DCs, T cells
CCR7	MIP-3β	B cells, T cells, DCs
CCR8	I-309	monocytes; macrophages
CCR9	CC chemokines	non-haematopoietic cells
Duffy antigen	IL-8, Groα, RANTES, MCP-1	erythrocytes
CX3CR1	fractalkine (neurotactin)	NK cells

Adapted from T.N. C. Wells and A.E.I. Proudfoot. Inflamm. res. 48 (1999) 353–362.

Interleukin-8- ***a key chemoattractant- and angiogenesis factor.***

IL-8 (MIP-2 in mice), is a potent chemoattractant, proliferative and angiogenesis inducing cytokine during inflammation (Ajuebor, Hogaboam et al. 2001). It is produced in high amounts in RA joints (Koch, Volin et al. 2001). IL-8R (CXCR2) deficient mice show defective neutrophil migration into sites of inflammation (White, Lee et al. 1998; Godaly, Hang et al. 2000). Synovial cells, mast cells, neutrophils, endothelial cells and macrophages at sites of inflammation produce IL-8. Blockade of IL-8 or IL8 receptor has been shown to reduce inflammation (Miura, Fu et al. 2001).

Macrophage chemokines and chemokine receptors.

MCP-1, MIP-1α/β and RANTES are chemoattractant to monocytes/macrophages and in some cases neutrophils (Yuan, Masuko-Hongo et al. 2001). The production of these mediators in the inflammatory milieu leads to recruitment and activation of monocytes/macrophages

through the C-C chemokine receptors CCR1, 2 and 5. The chemokines MCP-1 and RANTES act on chondrocytes leading to production of MMPs (Robinson, Scott et al. 2002). The inhibition of these chemokines leads to marked reduction in recruitment of monocyte/ macrophage and hence tissue damage. Antagonist to MCP-1 inhibited arthritis in MRL-lpr mice model (Gong, Ratkay et al. 1997). RANTES has been shown to be highly elevated in the chronic phase of a rat colitis model and antagonist Met-RANTES could inhibit inflammation (Ajuebor, Hogaboam et al. 2001).

1.7. Role of cell surface adhesion molecules in RA.

Leukocyte/endothelial cell adhesion molecules are essential mediators of both immune and inflammatory responses, involved in cell rolling, activation, adhesion and diapedesis (reviewed in Table 2).

Table 2.Cell adhesion molecules and function.

Step	Factors on endothelium	Factors on leukocytes
Rolling	P-selectin	PSGL-1
	E-selectin	ESL-1
	L-selectin ligand ?	Sialyl Lewis-X, CLA, L-selectin
Activation	Chemokines (IL-8, MCP-1), PAF	Cytokine and chemokine receptors
	PECAM-1	PECAM-1
	E-selectin	PSGL-1, ESL-1
Firm adhesion	ICAM-1, VCAM-1	β1, β2 and β7 integrins
Diapedesis	ICAM-1, VCAM-1, PECAM-1	β1, β2 and β7 integrins, PECAM-1

Taken from Szekanecz and Koch. Arthritis Research Vol 2 No 5.

Firm adhesion and emigration of rolling leukocytes such as neutrophils are mostly dependent on two members of the CD18 (β2)-integrin family; CD11a/CD18 [lymphocyte function-associated antigen-1 (LFA-1)] and CD11b/CD18 (Mac-1) on neutrophil surface and intercellular adhesion molecules (ICAM)-1 and ICAM-2 on the endothelium. ICAM-1 and VCAM-1 bind to integrins on the surface of leukocytes. The β2 integrin, lymphocyte LFA-1 is a monocyte/macrophage ligand for ICAM-1. Gene knockout studies suggest that absence of ICAM-1 significantly inhibits the development of arthritis and glomerulonephritis, while selectin deficiency results in accelerated development of joint and kidney inflammation (Bullard 2002). Studies also showed that β2 integrins may play a key role in regulating the initiation of psoriasis like skin diseases (Bullard 2002). Antibody and peptide inhibition

studies have suggested an important role for αvβ3 integrins in angiogenesis during inflammation (Maeshima, Yerramalla et al. 2001).

1.8. Role of leukocyte proteases and protease activated receptors in RA–a *final countdown to tissue damage.*

Leukocyte-derived proteases (Owen 1999) have the capacity to degrade every component of the extracellular matrix (ECM), and thereby play fundamental roles in physiological processes. In RA however the activity of these proteases is uncontrolled or deregulated contributing to tissue injury. The table. 3 reviews the major classes of leukocyte proteases involved in inflammation and tissue remodeling.

Table 3. Classification of leukocyte proteases according to catalytic mechanism.

Mechanism	Active site	pH optimum	Examples	Location
Serine proteases	Catalytic triad Asp, His, Ser	Neutral (pH 7–9)	Human leukocyte elastase	PMN, monocytes, mast cells, Eosinophils, basophils
			Cathepsin G	PMN, P monocytes
			Proteinase 3	PMN, P monocytes, mast cells
			Urokinase-type plasminogen activator	PMN, monocytes, macrophages
			Tryptase	Mast cells and basophils
			Chymase	Mast cells
			Granzyme A and B	Cytolytic T lymphocytes and natural killer cells
Metallo-proteases	Zn^{+2} coordinated to amino acids	Neutral (pH 7–9)	Interstitial collagenase (MMP-1)	Mononuclear phagocytes, eosinophils
			Neutrophil collagenase(MMP-8)	PMN, eosinophils
			72-kDa gelatinase (MMP-2)	Mononuclear phagocytes
			92-kDa gelatinase(MMP-9)	PMN, mononuclear phagocytes
			Stromelysin-1, -2, -3 (MMP-3,-10,-11)	Mononuclear phagocytes
			Matrilysin (MMP-7)	Monocytes
			Metalloelastase (MMP-12)	Macrophages
Cysteine proteases	Cys, His Acidic	(pH 3–6) At pH 7, cathepsin S retains 25% of its catalytic activity.	Cathepsin S	Lysosomes of most cells
			Cathepsin L	
			Cathepsin B	
			Cathepsin H	
Aspartic proteases	Asp (2 residues)	Acidic (pH 2–5)	Cathepsin D	Lysosomes of most cells

Adapted from Owen and Campbell , J Leuk Biol Volume 65, 1999

The serine proteases and metallo-proteinases play a major role in RA pathogenesis. Serine proteases such as elastase and cathepsin G hydrolyze proteins of ECM- Collagen, fibronectin and hyaluronan. The ECM breakdown products have chemotactic activity for neutrophils, monocytes, and fibroblasts (Horton, Shapiro et al. 1999; White, Livant et al. 2001). The serine

proteases tryptase, plasmin and thrombin produced by mast cells and neutrophils can activate cells by cleaving Protease activated receptors(PAR) (Asokananthan, Graham et al. 2002; Burysek, Syrovets et al. 2002; Frungieri, Weidinger et al. 2002). Inhibition of serine protease thrombin ameliorated CIA in mice (Marty, Peclat et al. 2001). The serine proteases (reviewed in table 4) of the complement system have an important role in complement activation and hence in inflammation mediated by autoantibodies.

Table 4: Serine proteases of complement activation pathway.

Protease	Activated by	Active form	Substrate
C1r	C1r	C1qr2s2	C1r and C1s
C1s	C1r	C1qr2s2	C2 and C4
MASP1	?	MBL–MASPs complex	?
MASP2	?	MBL–MASPs complex	C2 and C4
MASP3	?	MBL–MASPs complex	?
C2	C1s or MASP2	C4b2a	C3 and C5
Factor B	Factor D	C3bBb	C3 and C5
Factor D	Contact with substrate–cofactor complex	C3bBD complex	Factor B
Factor I	Contact with substrate–cofactor complex	Factor I complex with cofactor and C3b or C4b	C3b or C4b

Adapted from Biochemical Society Transactions (2000) Volume 28, part 5.

Matrix metalloproteinases (MMPs) are a group of zinc dependent endopeptidases that can degrade every component of the extracellular matrix. Deregulation of MMPs expression has been implicated in the pathogenesis of various diseases, such as arthritis, atherosclerosis, and tumor invasion and metastasis. A recent study showed that in mice MMP-2- deficiency results in severe arthritis, while MMP-9 deficient mice showed milder arthritis (Itoh, Matsuda et al. 2002).

1.9.Role of animal models in RA research– *outlook for human RA treatment.*

Animal models provide excellent opportunities to dissect mechanisms involved in RA pathogenesis. Though no single animal model for human disease can completely mimic a muti-factorial disease like RA, the simplicity of animal models means understanding the role of individual factors in the light of its genetic, environmental and pathogenic effects becomes

easy using gene knockout and in-vivo blocking/inhibition studies. Different animals models of same disease are valuable as each of them highlight different aspect of the disease e.g. some may highlight genetic factors, others may highlight effector mechanisms/or both. Hence there has always been a great demand for generation of new animal models for RA. Animals models have been used not only in disease hypothesis testing but as well in screening and efficacy testing of therapeutics drugs prior to human use. Table 5 reviews the current therapeutics for RA under development many of which are outcomes from animal experimentation and testing. Many of the drugs under development are targeted at innate immune mediators of inflammation.

Table 5: Current therapies under development for Rheumatoid Arthritis.

Company	Program
Abgenix (Fremont, CA)	Human Mab against IL-8
Aeterna Laboratories (Québec, Canada)	Angiogenesis inhibitor (AE-941) derivative for osteoarthritis and RA
Agouron (La Jolla, CA)	Selective matrix metalloprotease (MMP) inhibitor
Alexion (New Haven, CT)	Human Mab C5 inhibitor of the complement cascade
Amgen (Thousand Oaks, CA)	Oral tumor necrosis factor binding protein
Anergen (Redwood City, CA)	MHC peptide compound
AnorMed (Langley, BC, Canada)	Azaspirane immunomodulators (Atiprimod)
AutoImmune (Lexington, MA)	Synthetic type II collagen peptide (II-generation Colloral)
Axys Pharmaceuticals (S.Francisco, CA)	Cathepsin as arthritis target
BASF Bioresearch (Worcester, MA)	Anti-TNFα Mab
Bayer (Leverkusen, Germany)	Humanized anti-TNF antibody
Biogen (Cambridge, MA)	Recombinant human α-interferon
Biomatrix (Ridgefield, NJ)	Elastoviscous hylan biopolymer for osteoarthritis (Synvisc)
Boehringer Ingelheim (Ingelheim, Germany)	Antisense intercellular cell adhesion molecule-1 inhibitor
Boston Life Sciences (Boston, MA)	Oral amiprilose HCl (a modified hexose, Therafectin)
Cambridge Antibody Technology (UK)	Anti-TNF Mab
Celgene (Warren, NJ)	Thalidomide (Thalomid, formally Synovir)
Cell Genesys (Foster City, CA)	Human anti-IL-8 antibody from mouse transgenics
Centocor (Malvern, PA)	Chimeric anti-TNF Mab (Remicade)
Chiron (Emeryville, CA)	IGF-1 and IL-2
Cortech (Denver, CO)	Orally bioavailable neutrophil elastase inhibitor
Cypress Bioscience (San Diego, CA)	Protein-A matrix plasma apheresis column (Prosorba)
DepoTech (San Diego,CA)	IGF-1 and IL-2 DepoFoam formulations
Eli Lilly (Indianapolis, IN)	HSP-tetrapeptide to split IgA and α-antitrypsin
G.D. Searle & Co. (Skokie, IL)	COX-2 inhibitor celecoxib (Celebrex)
Genta (San Diego,CA)	Oral controlled-release formulation of diclofenac
IDEC Pharmaceuticals (San Diego,CA)	Second-generation anti-CD4 Mab
Immune Response Corp. (Carlsbad, CA)	Vβ3, 14 and 17 T-cell receptor therapeutic vaccine for RA
Immunex (Seattle, WA)	Soluble TNF receptor (Enbrel)
Inflazyme (Vancouver, BC, Canada)	Inflammatory cell activation inhibitor (Bispan)
Isis Pharmaceuticals (Carlsbad, CA)	Antisense intercellular cell adhesion molecule-1 inhibitor
Kaken Pharmaceutical (Kyoto, Japan)	Thrombin receptor-mediated signaling, NF-□B activation -RA
Ono Pharmaceutical (Osaka, Japan)	Orally bioavailable neutrophil elastase inhibitor
Peptide Therapeutics (Cambridge, UK)	HSP-tetrapeptide to split IgA and α-antitrypsin
SmithKline Beecham(Philadelphia, PA)	Second-generation anti-CD4 Mab
Supergen (San Ramon, CA)	IV pentostatin (small-molecule purine analog, Nipent)

Source:www.beunovista.com

2.1. Primary aims of the study.

i. Characterize the pathogenic GPI-specific auto-antibodies from the K/BxN murine model for Rheumatoid Arthritis.

ii. Dissect the roles of key innate immunity players in Rheumatoid Arthritis pathogenesis.

iii. Establish new murine models for Rheumatoid Arthritis.

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3.0. Materials and methods.

3.1. Materials.

3.1.1. General chemicals and Reagents-

All chemicals, reagents and solutions were of analytical grade and/or of highest commercially available purity. When not stated the chemicals were purchased from the Roth, Sigma, ICN or Merck. Microfuge tubes, centrifuge tubes, pipette tips were from Eppendorf (Hamburg), Biozym (Hess. Oldendorf) and TPP(Trassadingen, Swiss). ELISA plates were from TPP. BioMax Light X-Ray films were from Kodak.

3.1.2. Molecular biology reagents.

Bacterial strains XL2 Blue and TOP10F´were from Stratagene (Heidelberg) and BL21 was from Novagen (Heidelberg).

XL2 Blue MRF': Δ(mrcA)183 Δ(mcrCB-hsdSMR-mrr)^{173} endA1 supE44 thi-1 recA1 gyrA96 relA1 lac[F' proAB lacI^{q}ZΔM15 Tn10 (Tet^{r}) Amy Cam^{r}]^{a}.

TOP10F': F'{lacI^{q} Tn10(Tet^{R})}mcrA (mrr-hsdRMS-mcrBC) Φ80lacZ M15 lacX74 recA1 deo^{R} araD139 (ara-leu)^{7697}galU galK rpsL (Str^{R}) endA1 nupG.

BL21(DE3): F^{-} ompT hsdS$_{B}$(r$_{B}^{-}$m$_{B}^{-}$) gal dcm (srl-recA)306::Tn10(DE3).

Restriction enzymes and buffers were from Fermentas and Promega. *Taq* and *Pfu* DNA polymerase, T4 DNA ligase and Alkaline phosphatase were from Gibco BRL(Eggenstein). TA TOPO Cloning^{R} kit was from Invitrogen (Groningen, Netherlands). cDNA synthesis kit was from Gibco BRL. DNA MW markers were from Gibco BRL. Plasmid mini extraction kits were from Amersham. Midi plasmid extraction kit and DNA gel extraction kit were from Qiagen (Munich). RNA extraction reagent RNAzol^{R} was from WAK-Chemie(Bad Soden). GTG low melting agarose was from Biozym. Oligonucleotides for PCR were from MWG-Biotech (München). DNA sequencing was done by GATC (Konstanz).

3.1.3. Antibodies.

Anti mouse CD4-PE and anti-Vβ6 antibodies were from BD Pharmingen (Heidelberg). Rabbit anti-mouse kappa light chain and rabbit anti-mouse lambda light chain were from BD pharmingen. Secondary antibodies goat anti-mouse antibody-HRP and Goat anti-rabbit antibody-HRP were from Pierce (Rockford, USA).

3.1.4. Cell culture reagents.

Iscove´s modified Dulbeccos Eagle medium for cell culture and Hybridoma-SFM media, fetal calf serum, penicillin, streptomycin, glutamine, HAT and PEG were bought from Gibco BRL. Cell culture plates, serological pipettes and confocal dishes were obtained from Costar and Greiner (Frickenhausen).

3.1.5. Protein chemistry reagents.

Acrylamide solutions for protein gels were from Roth. Protein gel MW markers were from Amersham(Frieburg) and prestained markers from Invitrogen. Protease inhibitor cocktail was from Boehringer (Mannheim). Nitrocellulose membranes were from Schleicer and Schuell. Enhanced chemiluminescent reagent was from Pierce. GST bulk purification module and Hi Trap Protein G column were from Amersham. Epoxy magnetic beads for antibody crosslinking were from Dynal Biotech. Trypsin and Glu-C were from Promega. Microcon concentrators were from Millipore.

3.1.6. Experimental animals.

KRN TCR mice on C57Bl/6 background (Kouskoff, Korganow et al. 1996) was a kind gift from D. Mathis and C. Benoist, IGMBC France, and maintained and bred at animal facility of University Konstanz.

BALB/C, C567BL6 and NOD/lt mice were from animal facility University Konstanz.

Cr2$^{-/-}$ (Chen, Koralov et al. 2000) and *C4*$^{-/-}$ (Fischer, Ma et al. 1996) mice are a kind gift from M.C. Carroll, Boston, USA and were maintained and bred at animal facility, University Konstanz.

FcγRIII$^{-/-}$ (Hazenbos, Gessner et al. 1996) and *FcγRIIb*$^{-/-}$ (Takai, Ono et al. 1996) mice on mixed (B6 x 129) background were from Jackson laboratory,USA; bred and maintained at animal facility, University konstanz.

TNFR1$^{-/-}$ (Pfeffer, Matsuyama et al. 1993) and *TNFR2*$^{-/-}$ (Erickson, de Sauvage et al. 1994) mice were a kind gift from Dr.Wendel, University Konstanz

Chinchilla rabbits for anti-sera production were from animal facility, University konstanz.

3.1.7.Therapy reagents.

Cromolyn, Cimetidine, and Mepyramine, were from Sigma (Deisenhofen), SB225002 from Calbiochem, Tranilast was from Biotrend, sheep anti- mouse TNFα polyclonal antibodies was a gift from Dr.Wendel, University konstanz, polyclonal anti-mouse MCP-1 and anti-mouse

MIP-2 antibodies were from DPC Biermann (Germany), Cobra venom factor was from Quidel biotech. Clodronate liposomes was a gift from N. Van Roojen Vrije University, Holland.

3.2. Methods.

3.2.1. Recombinant DNA techniques.

3.2.1.1. Preparation of bacterial media.

Bacterial culture media-LB (Luria-Bertani)-Medium, LB agar, NZYM-Medium und SOC-medium were prepared according to Maniatis *et al*.1991.

3.2.1.2. Isolation of RNA.

RNA was extracted by RNAzol B reagent. Cells/tissue was homogenized with 2 ml RNAzol B per 100 mg tissue or 10^7 cells. Phase separation was done by adding 0.2 ml of chloroform per 2.0 ml homogenate and spun at 12,000g for 15 min at 4^{o}C. The upper colorless aqueous phase was transferred into clean tube and RNA precipitated by adding equal volume of isopropanol and spinning at 12,000g for 15 min. The pellet washed 75% ethanol, air dried and suspended in DEPC water. The quality of the RNA was verified by gel electrophoresis.

3.2.1.3. cDNA synthesis and polymerase chain reaction (RT-PCR).

cDNA synthesis was done according to manufacturer protocol. Briefly 5 μg of total RNA with 1 μl (50 μg) primer oligo(12-18) dT in volume of 12 μl DEPC water was incubated at 70^{o}C for 10 min. 7 μl of reaction mix solution (10X PCR buffer, 25mM $MgCl_2$, 10 mM dNTP mix and 0.1M DTT) was mixed with RNA primer mix and incubated at 25^{o}C for 5 min and for 10 min after adding 1 μl (200U) Superscript II reverse transcriptase enzyme. RT reaction was carried out at 42^{o}C for 50 min and terminated at 70^{o}C for 15 min. RNA was removed by treatment with 1 μl RNAse H at 37^{o}C for 15 min. The cDNA quality was checked by GAPDH PCR. For general PCR, *Taq* DNA polymerase and for PCR cloning, *Pfu* DNA polymerase was used. A standard 50 μl PCR mix consisted of 5 μl of 10X PCR buffer, 1 μl of 0.25-1 μg of template, 5 μl of 10 μM primers each, 2 μl of 10 μM dNTP mix, 3 μl of 1.5 mM $MgCl_2$. The standard PCR cycling conditions were- an initial denaturation at 95^{o}C for 2 min, followed by 30 cycles of denaturation at 95^{o}C for 45 sec, annealing at 50^{o}C to 60^{o}C for 1 min and extension of 72^{o}C for 1 min/Kb pair for *Taq* polymerase and 2 min/Kb pair for *Pfu* polymerase extension reaction. A final extension reaction was done at 72^{o}C for 5 min.

3.2.1.4. Addition of 3´adenosine to PCR product.

Prior to TOPO cloning of *Pfu* DNA polymerase amplified PCR products addition of poly A ends was done by adding 1 *µl* of Taq pol (3U/ *µl*) and 1 *µl* of 10mM ATP to 50 *µl* PCR mix and incubation at 72^{o}C for 10 min.

3.2.1.5. TOPO cloning of PCR product

The TOPO TA cloning of PCR amplified products into pCR2.1-TOPO vector were done according to manufacturers recommendations. The ligated products were transformed into T0P10F´ cells and screened by blue white selection.

3.2.1.6. Extraction of plasmid-DNA.

Plasmids preps from 1-3 ml cultures were prepared using GFX^{TM} Micro Plasmid Prep Kit. Bacterial cells from 1.5 ml cultures was suspended in 150 *µl* solution I (100 mM Tris-HCl (pH7.5), 10 mM EDTA, 400 *µg* /ml RnaseI) and lysed in 150 *µl* alkali solution II (1M NaOH, 5.3% SDS) and then neutralized by 300 *µl* solution III (buffered solution containing acetate and chaotrope). The lysate was pelleted and supernatant applied to glass fiber GFX^{TM} column and washed with wash buffer (Tris-EDTA buffer with 80% ethanol). Plasmid was eluted by Tris-EDTA buffer pH 8.0. The Qiagen plasmid midi kit was used for plasmid extraction from 25-50 ml cultures. Overnight culture was diluted 1/500 in 25 ml medium and grown at 37^{o}C for 12-16 h. The bacterial cells were suspended in 4 ml buffer P1 (50 mM Tris-HCl ,pH 8.0, 10 mM EDTA, 100 *µg* /ml Rnase A), alkali lysed by 4 ml of buffer P2 (200 mM NaOH, 1% SDS) and then neutralized by buffer P3 (3.0 M Potassium acetate). The lysate were pelleted and supernatant loaded on equilibrated Qiagen-tip 100 column to bind DNA. The columns were washed by wash buffer (1M NaCl, 50 mM MOPS, pH7.0, 15% Isoproponal *v/v,* 0.15% Triton X-100) and plasmids were eluted with 5 ml elution buffer (1M NaCl, 50 mM MOPS pH 7.0, 15% Isopropanol *v/v*). The plasmid was precipitated by 0.7 volumes of isoproponal and by centrifugation at 15000g for 30 min, The DNA pellet was washed in 70% ethanol, dried and dissolved in 10 mM Tris Cl pH 8.0.

3.2.1.7. Restriction digestions of plasmid DNA.

Upto 5 *u*g plasmid DNA was suspended in 20 *µl* of restriction enzyme buffer. 1-5 units of restriction enzyme was added and reaction mix incubated at 37^{o}C for 2h-4h. The reaction was stopped by 0.2 *µl* 500 mM EDTA pH8.0. The restriction digests was analyzed by gel electrophoresis.

3.2.1.8. Isolation of DNA and plasmids from preparative agarose gel.

Purification of plasmid and DNA inserts was carried out on 0.8-2% low melting point agarose gels. EtBr stained gels were visualized on a UV trans-illuminator (λ 265 nm). A clean scalpel was used to excise the insert bands of interest and DNA was extracted using the QIA quick gel extraction kit. Briefly 3 volumes of buffer QG was added to 1 volume of gel and incubated at 50°C for 10 min followed by 1 gel volume of Isopropanol. The mix was applied to QIA quick column to bind DNA and washed with buffer PE. The DNA was eluted with 10 mM Tris-Cl, pH 8.5.

3.2.1.9. DNA ligation.

For ligation reaction 1U of T4 ligase was added in 1:3 molar ratio of plamid to insert DNA (0.5-1 *μg* total DNA) in 20 *μl* 1X ligase buffer mix. The reaction was incubated at RT for 3-5 hours. 2-4 *μl* of reaction mix was used to transform 100 *μl* competent cells.

3.2.1.10. Preparation of competent bacteria.

Single colony from LB plate without antibiotic was picked and inoculated into 100 ml SOCS medium. The culture was grown at 37°C until an OD (λ600nm) of 0.5. The bacteria were pelleted at 4000 rpm and resuspended in ice-cold 50 mM $CaCl_2$ and incubated on ice for 30 min. The $CaCl_2$ treated bacteria were pelleted again at 2600g at 4°C for 5 min and carefully resuspended in 4 ml of 50 mM $CaCl_2$. The bacteria were aliquoted and stored at -70°C until use.

3.2.1.11. Transformation of competent bacteria.

The competent cells were thawed on ice and mixed with 0.1-2 *μg* of purified plasmid DNA or with ligation mix and incubated on ice for 30 min. Subsequently the cells were heat shocked at 42°C for exactly 45 sec and incubated on ice for 2 min. The cells were suspended in 200 *μl* SOCS media and incubated for 45 min at 37°C with shaking. 10-200 *μl* of the transformation mix was plated onto petri plates with selection medium and incubated overnight at 37°C.

3.2.2. Protein techniques.

3.2.2.1. Gel electrophoresis of protein.

SDS-polyacrylamide-gel electrophoresis was performed according to Laemmli. Gels of different percentages were casted with acrylamide/bisacrylamide (30:0.8) depending on the size of the investigated protein. All samples were supplemented with 0.25x vol. sample buffer

(100 mM Tris, pH 8.0, 25% SDS, 50% β-mercaptoethanol, 0.5% bromophenolblue), and denatured at 95°C for 5 min and loaded. Gels were run with a constant current of 80 mA/gel in Laemmli buffer (25 mM Tris, 192 mM Glycine, 0.1% SDS). Staining of gels were done with Coomassie stain (40% methanol, 10% acetic acid, 0.2% Brilliant Blue R250). The gels were destained with destaining solution (40% methanol, 10% acetic acid).

3.2.2.2. Expression and purification of GST fusion recombinant proteins.

10 ml of LB ampicillin media was inoculated with recombinant protein expressing BL21(DE3) *E.coli* and grown overnight (o/n) at 37°C. 5 ml from o/n culture was used to inoculate 200 ml LB ampicillin media in 1litre flask and shaken at 37°C for 2-3 hours until OD λ 600 nm was 0.6. The cultures were induced with 1 mM IPTG (end concentration) and shaken at 37°C for 3 hours to express the protein. The cells were pelleted at 3500 rpm in sorvall GSA rotor for 30 min at 4°C. For every gram of cell pellet, 3 ml of ice cold lysis buffer (50mM Tris pH 8.3, 100mM NaCl, 1mM EDTA pH 8.0), 8 *μl* of 50mM PMSF, 125 *μl* of 25X Protease inhibitor cocktail and 80 *μl* of lysozyme (10mg/ml) were added and incubated on ice for 20 min with occasional mixing. 4 mg of deoxycholic acid was then added to lysate with stirring at 37°C until the lysate became viscous. To this lysate 20 *μl* of DNAseI (1mg/ml) was added and placed at RT until viscosity was lost (30 min). The lysate was then sonicated on ice with at micro-tip limit for 10 times (30 sec intervals each) or until OD λ600 nm was 1/5 the initial. The sonicate was pelleted at 10,000 rpm in sorvall F6 rotor for 20 min at 4°C and supernatant collected. 1 ml Glutathoine beads for every for 5 mg GST-fusion protein (yield from approximately 2 liter of culture) was re-suspended in 1ml lysis buffer and the sonicate supernatant added to beads and mixed at RT for 30 min. The beads were pelleted at 500g for 5 min, supernatant removed and were washed three times with 10 bed volumes of 1X PBS, 0.5% Triton X. The recombinant GST-fusion protein was cleaved off from beads incubated overnight at RT after adding 10 U of Thrombin protease/mg of recombinant protein. The cleaved GPI was eluted from beads and checked on SDS-PAGE for purity.

3.2.3. Immunological techniques.

3.2.3.1.Western blots and dot blots.

After protein gel electrophoresis. Two layers of Whatman 3MM paper, nitrocellulose membrane, the gel, and two more layers of 3MM paper all soaked in blotting-buffer (20% methanol, 48 mM Tris, 39 mM glycine), were stacked onto the anode of a semi-dry-blotter. Air bubbles between the layers were removed by rolling a glass pipette over the sandwich.

Proteins were transferred at 2.75 mA/cm^2 for 75 min. After the blotting, complete transfer and equal loading of the proteins were controlled by staining the gel with Coomassie and the membrane with Ponceau red (0.2% Ponceau S, 5% acetic acid). The membranes were then incubated at RT for 1 h or at 4^{o}C overnight, in TBST (150 mM NaCl, 50 mM Tris/HCl pH 8, 0.05% Tween 20), 5% non-fat dry milk to block non-specific binding of the antibody. Excess milk protein was removed by several washing steps with TBST. The membrane was incubated at RT for 1h with primary antibody diluted in TBST. After further washing in TBST (3x 5 min), the membrane was incubated at RT for 1h with secondary antibody. This was a goat anti-mouse HRP (1:10,000 in TBST) or a goat anti-rabbit HRP (1:5000 in TBST). After another three washing steps, HRP activity was detected by Enhanced Chemi Luminescence (ECL) on X-ray film. For dot blots the proteins were spotted on nitrocellulose membranes and processed in same manner as mentioned for immunoblots for antibody staining.

3.2.3.2. GPI Enzyme-Linked Immunosorbent Assay (ELISA).

Purified, GST-cleaved GPI was coated (0.125 μg/well in PBS, pH 7) onto 96 well ELISA microtiter plates. The nonspecific binding sites were blocked with 1% fat-free milk powder/PBS. Sera from K/BxN serum-injected mice as well as the control serum-injected mice were diluted 1:300 in PBS with 1% milk powder and added to the wells. After incubation for 2 h at 37^{o}C the plates were washed with PBS/Tween 20 and incubated for 2 h with rabbit anti-mouse-IgG-HRP antibodies (1:20,000). The plates were developed with ortho-phenylene-diamine as substrate. The reaction was stopped by adding 1M HCl and read at 495nm OD.

3.2.3.3. Preparation of antigen for immunization.

For mice immunization 250 μg of recombinant GPI was dissolved in 1 ml of 5% Alum (Aluminum potassium sulphate) as adjuvant. Then the pH was then shifted to pH 6.5 using 1.25 N NaOH to induce precipitation of GPI-alum complex. Each mice was immunized by injecting 75 μg (300 μl) of the antigen-adjuvant mixture intra-peritoneally. For rabbits immunization with 200 μg of recombinant GPI plus 500 μl of complete Freunds adjuvant for primary dose or in 500-µl incomplete Freunds adjuvant for booster dose in a total volume of 1 ml saline was injected sub-cutaneously.

3.2.3.4. Production of GPI specific monoclonal antibodies.

A week prior fusion SP2/0 myeloma fusion partner cells were expanded in Iscoves medium 10% FCS, 96 well plates were plated with peritoneal cells obtained from C57Bl6 mice and the KBN mice were immunized with purified recombinant GST cleaved GPI. On day of fusion the mice were sacrificed; spleens harvested and teased to obtain a single cell suspension. Debris were removed by passage through fine steel mesh and washed cells were suspended in serum free Iscoves media. The spleen cells were mixed in 1:1 ratio of SP2/0 in 50 ml conical centrifuge tube, pelletted at 500g and the supernatant discarded. The cell fusion was performed in laminar hood with cell pellet in 37^{o}C water bath. Using a 1 ml pipette 1 ml prewarmed 50% PEG solution was added drop-by-drop over 1 min with stirring with pipette after each drop. This was repeated with additional 1 ml of prewarmed serum free Iscoves medium. A further 7 ml of prewarmed serum free Iscoves medium was added drop-by-drop over 2-3 min. The fusion mix was pelletted at 500g for 5 min at RT and supernatant discarded. Placing the cell pellet at 37^{o}C, 10 ml of prewarmed Iscoves medium with 20% FCS was forcibly added to disrupt the cell pellet and the volume made to final cell density of 2.5x10^{6} cells/ml. The cell suspension is then gently pipetted out at 100-125 μl /well into a 96 well flat bottom plate with feeder cells and incubated in 37^{o}C, 5% CO_2. On days 2,3,4,5,7,9, and 11 half the volume of each well was aspirated using sterile Pasteur pipette attached to vacuum pump and 2 drops of Iscoves with 20% FCS, and 1X HAT was added. On day 14 the feeding was done with Iscoves with 20% FCS and 1X HT. From day 15 onwards the cells were fed with Iscoves medium 20% FCS. The hybridomas were screened when the cells showed 10%-25% confluence. About 100 μl supernatant from each well were removed and tested for GPI reactivity by GPI ELISA.

3.2.3.5. Limited dilution cloning of hybridomas and adaptation to serum free media.

10 days prior to cloning, 96 well plates were plated with peritoneal cells obtained from C57BL/6 mice and grown at 37^{o}C. On the day of cloning hybridoma cells were counted and checked for viability by trypan blue dye exclusion stain. The viability must be atleast 80% for successful cloning. For each hybridoma, the cells were serially diluted in Iscoves medium with 20% FCS to give 4, 2 and 1 cell/ml. The diluted cells were then plated at 250 μl /well for each dilution in prepared 96 well plates and incubated at 37^{o}C, 7.5% CO_2 for 5-7 days. The plates were microscopically examined for plating and cloning efficiency. Wells with single clones were followed up for GPI reactivity by ELISA and positive clones were expanded for freezing. The hybridomas were adapted to grow in serum free media (SFM) to reduce protein

loads in downstream processing. Briefly the hybridoma was grown in Iscoves media 10% FCS. The growing cells were then serially expanded in Iscoves media 10% FCS with 25% SFM followed by 50% SFM medium and finally in complete 100% SFM media.

3.2.3.6. Purification of monoclonal antibodies.

The hybridomas were grown in hybridoma medium to confluency and the medium turned yellow. The supernatants were collected and concentrated 10 times using an Amicon concentrator (Millipore) having a membrane cutoff 100 KDa. The purification of antibodies from the concentrated hybridoma supernatants was done on AKTA^{TM} Prime instrumentation (Amersham) using Hi Trap Protein G columns. Briefly concentrated supernatants were loaded onto Protein G columns equilibrated with binding buffer (20 mM sodium phosphate pH 7.0). The columns were washed with binding buffer to remove non-specific proteins. The antibody was then eluted with elution buffer (0.1 M Glycine-HCl, pH 2.7). The eluted antibody was immediately neutralized with 1M Tris pH 9.0 and buffer exchanged into PBS buffer using a microcon-50 concentrator (Millipore).

3.2.4. Epitope analysis by affinity proteomics.

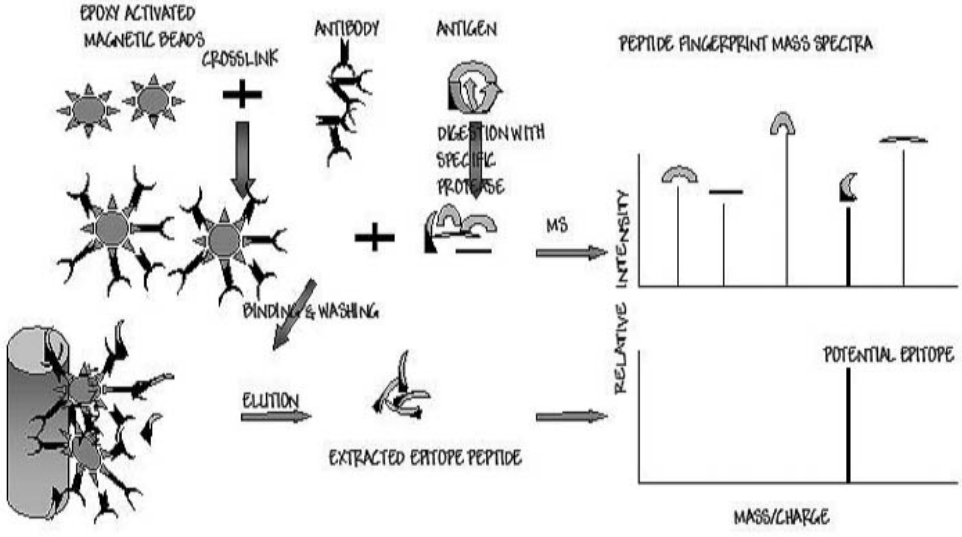

Figure 5: Illustration showing principle of Mass spectrometry based epitope analysis.

Rapid developments in mass spectrometry in combination with the proteolytic digestion of antigen have opened new methods of epitope mapping (Macht, Fiedler et al. 1996; Parker, Papac et al. 1996). The method is based on the fact that antibody in general are very resistant

to proteolytic digestion. The epitope analysis can be done by epitope excision or extraction In epitope excision immobilized monoclonal antibody is incubated with whole antigen and the bound antigen is digested with a proteolytic enzyme The antibody bound surface of the antigen is protected from proteolysis. In epitope extraction protease digested antigen peptide mixture is affinity bound to immobilized antibody. In both the above cases the peptides containing the epitopes are eluted from immobilized antibody by low pH buffer and analyzed by mass spectrometric peptide fingerprinting. The high accuracy and sensitivity of mass spectrometer helps in identifying the peptides by molecular weights.

3.2.4.1. Cross linking of GPI antibody to epoxy dynabeads.

Dynabeads® M-270 Epoxy are uniform, super paramagnetic beads with surface reactive epoxy groups for binding of antibody or other ligands by covalent bond formation at neutral pH. Binding of antibodies occurs through amine or thiol groups occur with no further activation of the surface. For antibody cross-linking, the beads were washed and resuspended in 0.1 M sodium phosphate buffer pH 7.4 at 10^9 beads/ml. 3 μg of antibodies/10^7 beads in phosphate buffer, 1 M ammonium sulfate (to enhance binding of antibody) were mixed, incubated for 24 hours at RT with gentle mixing. After incubation the tube with the beads were placed in magnet and supernatant removed. The beads were washed with PBS buffer and stored at 2-8^{o}C.

3.2.4.2. Proteolytic digestion of purified recombinant mouse GPI.

Digestion of GPI was carried with Trypsin or Glu-C protease for peptide fingerprinting western blot of monoclonal antibodies and for epitope analysis by mass spectrometry**.** Trypsin is a serine protease, which cleaves peptide bonds C-terminally of arginine or lysine. Trypsin digestion of GPI was done at a enzyme/substrate ratio of 1/50 in 0.1 M Tris HCl buffer pH 8.0 containing 0.01% *w/v* SDS at 37^{o}C for 12 hours. The reaction was stopped by adding 0.1% TFA. Glu-C is a serine protease, which cleaves peptide bonds C-terminally to glutamic acid. The digestion of GPI was done at an enzyme substrate ratio of 1:50 in 25 mM ammonium carbonate buffer pH 7.8 containing 0.01% w/v SDS at 25^{o}C for 12 hours.

3.2.4.3. Epitope extraction.

For epitope extraction 10^8 Dynabeads immobilized with GPI monoclonal antibodies were incubated with 50 μg peptides of Trypsin or Glu-C digested mouse GPI in 1 ml PBS buffer, pH 7.0 for 4 hours with shaking. The beads were washed 10X with 1 ml PBS, 0.1% Tween on

a magnet. The peptides were eluted with 50 *µl* of 0.1% TFA solution. The eluates were concentrated in a speed vac and resuspended in 0.1% TFA for mass spectrometric analysis.

3.2.4.4. Mass spectrometry analysis.

MALDI time-of-flight mass spectrometry was performed with a Bruker Biflex-DE mass spectrometer equipped with a Scout MALDI source and video system, a nitrogen UV laser (λ_{max} 337 nm), dual-channel plate detector and XMASS software. Sample preparation was performed with 1 *µl* of a freshly prepared saturated solution of 4-α-hydroxy cyanocinnamic acid in 0.1% TFA in H_2O and 0.1% TFA in Acetonitrile (*v/v* 1:2), which was directly mixed with 0.5 *µl* of the peptide solution and crystallized on targets. Spectra were recorded at an acceleration voltage of 25 kV and were averaged over 5 single laser shots. Calibration was carried out using the singly and doubly protonated ions of bovine insulin as internal standard.

3.2.5. Animal experimentation.

3.2.5.1. Establishment of KBN mice model and production of KBN sera.

Arthritic mice were obtained by crossing KRN-TCR transgenic maintained on C57BL/6(K/B) with NOD/Lt (N) animals (K/BxN). Progeny bearing the Vβ6 transgenic TCR were identified by cytofluorometry of PBL using anti-CD4 PE and anti-Vβ6 FITC labeled Abs.

3.2.5.2. Establishment of antibody induced arthritis in mice.

Sera were obtained by tail bleeding of K/BxN mice aged 1-6 months. Sera were pooled and stored at –20^{o}C until use. 100 *µl* equivalent of sera was injected *i.p* per mice to induce arthritis on day 0 and in some cases again on day 10 to prolong inflammation. For GPI monoclonal induced arthritis 4.0 mg of total GPI mAb cocktail in a volume of 200 *µl* saline was injected *i.v* per mice on day 0 followed injection of 50 *µg* LPS (*E.coli* strain 0111B4) *i.p*. on day 1. For Rabbit anti- mouse GPI sera induced arthritis 300 *µl* of rabbit anti-sera was injected *i.p* per mice to induce arthritis.

3.2.5.3. Assessment of antibody induced arthritis by ankle thickness and clinical index score.

Development of RA in the mice was assessed by calliper (Mitutoyo, Japan) measurement of ankle thickness and by clinical index score-a visual score based on number of ankles affected. Ankle thickness for each mice was expressed in millimeters as summed average of thickness of the four limbs of each mice. The error was expressed as standard error from mean (SEM).

Clinical Index(I) score was obtained by summing of ankles scores: Ankle score is 0 if ankle not affected; 1 if ankle affected and 2 if 2 ankles affected and so on. Clinical index(II) score was obtained by sum of multiplied values of each ankle score with its inflammation score. Ankle score is 0, if ankle not affected and 1, if ankle affected. Inflammation score is 0 ankle not affected; 0.25 if reddening and slightly swollen ankle; 0.75 if reddening and moderately swollen ankle; 1 if reddening and highly swollen ankle. The error was expressed as standard error from mean (SEM).

The percentage reduction in maximum ankle thickness was obtained by formula [1-(maximum average ankle thickness of treated mice/ maximum average ankle thickness of non- treated mice] x 100. The percentage reduction in clinical index was calculated similarly.

3.2.5.4. Treatments protocols for and inhibition studies.

3.2.5.4.1. Complement depletion by cobra venom factor treatment.

Cobra Venom Factor was injected into mice to deplete complement C3 and C5. A single *i.p* injection of 500U CVF/Kg body weight was done for this purpose. Stock solution of 3U/ *µl* in saline were prepared and stored at -70^{o}C and were diluted 10 times with saline prior to injection.

3.2.5.4.2. Depletion of macrophages.

Macrophages were depleted by Clodronate liposomes treatment (Van Rooijen and Sanders 1994; van Rooijen, Sanders et al. 1996). Two *i p.* injections of 200 *µl* of liposomes were done on day day-3 and -1 prior to sera transfer to deplete macrophages.

3.2.5.4.3. In vivo inhibition of mast cell degranulation.

For mast cell degranulation inhibition with cromolyn (Kolaczkowska 2001), mice were treated with Cromolyn sodium 10mg/mice/injection to inhibit Mast cell degranulation. A 50 mg/ml solution of Cromolyn was prepared in saline. Three *i.p* injection, of 10 mg each (200 *µl*) were made on day -1, 0 and 2 with regard to sera transfer. 100 *µl* of KBN sera was injected *i.p* 2 hours after the day 0 cromolyn treatment. For Mast cell degranulation inhibition with tranilast, 2-[[3-(3,4-Dimethoxyphenyl)-1-oxo-2-propenyl]amino] benzoic acid.(Hara, Ono et al. 2002), mice were treated with tranilast 250 *ug*/mice/injection. A solution of 2mg/ml solution of tranilast was prepared in saline. Three *i.p* injections of 250 *µg* each (125 *µl*) were made on day -1, 0 and 1 with regard to sera transfer. 100 *µl* of KBN sera was injected *i.p* 2 hours after the day 0 treatment. The mice were assessed for arthritis development.

3.2.5.4.4. Administration of histamine receptor antagonists.

Mepyramine, a H1 histamine receptor antagonist or cimetidine, a H2 histamine receptor antagonist(Kolaczkowska 2001) were administrated at 450 *ug*/mice/injection to study the role of histamine and its receptors. A solution of 5mg/ml mepyramine was prepared in saline. Cimetidine base was dissolved in a small quantity of 0.1 N HCl, neutralized with NaOH, and made to 5mg/ml in saline. Three *i.p* injections of 450 *ug* each (90 *µl*) were made with mepyramine or cimetidine into each mice on day -1,0 and 2 with regard to sera transfer. 100 *µl* of KBN sera was injected *i.p* 2 hours after day 0 treatment. The mice were assessed for arthritis development.

3.2.5.4.5. Administration of IL8R antagonist.

The compound SB225002-a small molecule IL8 receptor-b (CXCR2) antagonist (White, Lee et al. 1998) was administered at 80 *µg*/mice/injection. A solution of 400 *µg*/ml SB225002 was prepared in 25% DMSO, saline. Four *i.p* injections of 80 *µg* each (200 *µl*) were administered on day -1, 0, 2 and 4 with regard to sera transfer. 100 *µl* of KBN sera was injected *i.p* after 30 min of day 0 treatment. The mice were assessed for arthritis development.

3.2.5.4.6. Administration of blocking antibodies.

i. Rabbit anti-mouse/rat Macrophage inflammatory protein-2 (MIP-2) antibodies were administered at 150 *ug*/mice/injection. Two *i.v* injections of 150 *µg* each (150 *µl*) were made with the antibodies resuspended in saline on day 0 and 3 with regard to sera transfer.

ii. Rabbit anti-mouse/rat Macrophage Chemoattractant Protein-1 (MCP-1) antibodies were administered at 150 *ug*/mice/injection. Two *i.v* injections of 150 *µg* each (150 *µl*) were made with the antibodies re-suspended in saline on day 0 and 3 with regard to sera transfer.

iii. Sheep anti- mouse TNFα were administered at 500 *µg* /mice/injection. Two *i.v* injections of 500 *µg* each (100 *µl*) were made with the antibodies re-suspended in saline on day 0 and 3 with regard to sera transfer.

3.2.6. Histochemical techniques.

Mice were sacrificed by cervical dislocation .Tissue samples were prepared by fixing tissues 24 h in 4% phosphate-buffered formaldehyde. Fixed joints were decalcified by treatment with 14% EDTA solution for 2 weeks with gentle rocking and daily replacement of the solution. Samples were then washed with PBS, dehydrated with a series of ethanol washes (50%

ethanol, followed by 70% ethanol), and embedded in paraffin. Sections of tissue 4 µm thick were cut by microtomy and stained with haematoxylin and eosin.

4. Results.

4.1. Generation and characterization of glucose-6-phosphate isomerase specific monoclonal antibodies from K/BxN mice.

GPI specific antibodies from K/BxN mice are arthritogenic and can transfer arthritis into naive mice. The reason for the arthritogenicity nor the joint specificity and cross reactivity of GPI antibodies is still not known. And moreover a comparative study between the auto-antibodies in the K/BxN murine RA model and to that in human RA patients would be interesting. For these reasons it was decided to elucidate the epitopes recognized by the GPI specific pathogenic antibodies from the K/BxN mice. GPI-specific ELISA and immuno-blotting screening assays were needed to generate the GPI reactive hybridomas from K/BxN mice. Therefore full-length recombinant mouse GPI was cloned expressed and purified in bacteria and mouse GPI specific ELISA and immunoblots screening assays established. Also the truncated versions of mouse GPI proteins were cloned and expressed in bacteria for use in epitope mapping investigations of the GPI antibodies. To study the possibility that the RA patients had similar autoantibodies to GPI as in K/BxN mice, human GPI specific ELISA and GPI immunoblot screening assays were setup from the recombinant human GPI cloned expressed and purified from bacteria.

4.1.1. Cloning, expression and purification of recombinant mouse glucose-6-hosphate isomerase.

The mouse full length GPI was cloned by RT-PCR from total C57BL6 thymus RNA using forward primer, KN143 -5´GGA GAA TTC TAT GGC TGC GCT CAC CCG GAA 3´ and reverse primer KN144 -5´TTC CTC GAG TCC CAA CAG CCT TGT AGT CC 3´. The primers had EcoRI and XhoI recognition sites for in-frame cloning into pGEX-4T-3 GST fusion vector. *Pfu* DNA polymerase was used for error free amplification of the product. The PCR cycling conditions were 94^{o}C for 2 min, followed by 30 cycles of denaturation at 94^{o}C for 45 sec, annealing at 60^{o}C for 1 min and extension at 72^{o}C for 4 min, followed by a final extension reaction at 72^{o}C. Figure 6 shows the PCR amplified full-length mouse GPI amplicon.

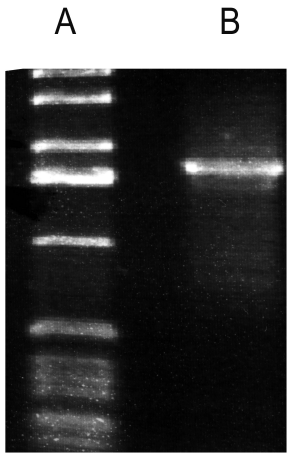

Figure 6. PCR amplification of mouse GPI. (A) Marker. (B) Amplified PCR mGPI gene product.

The PCR product was cloned into TOPO vector; transformed into TOP10F´ *E.coli* and recombinant clones selected by blue/white screening. The insert DNA were excised from recombinant vectors by EcoRI and XhoI digestion, separated on low melting point agarose gel, extracted and sub-cloned into pGEX-4T-3 GST fusion vector. Positive clones with the right size DNA insert (figure 7) were further amplified and sequenced.

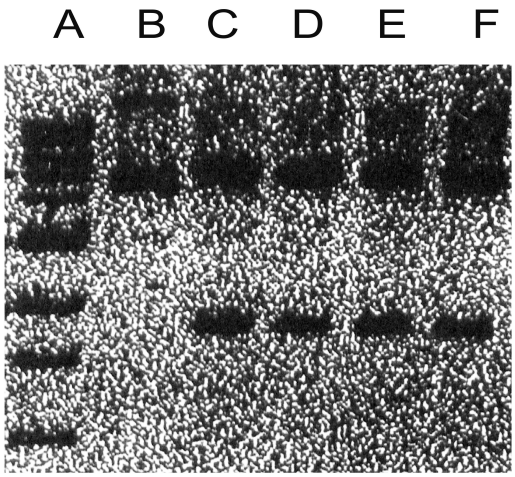

Figure 7. Screening of pGEX-4T-3 plasmids for recombinant mouse GPI inserts by restriction enzyme digestion analysis. (A) Marker. (B) Plasmid without insert. (C-F) Plasmids with insert.

Recombinant plasmids with mouse GPI inserts were confirmed by DNA sequencing and transformed into BL21 *E.coli* for protein expression. The bacterial clones were screened by SDS-PAGE for recombinant protein expression on IPTG addition. For purification of GPI protein the recombinant BL21 *E.coli* were grown to log phase and the protein was induced with IPTG. The bacterial cells were lysed, sonicated and the supernatant containing the recombinant GST-GPI fusion protein was affinity purified on Glutathione sepharose beads. The recombinant mouse GPI was cleaved from the beads using thrombin protease. The purity of the cleaved mouse GPI protein was checked by SDS-PAGE. Figure 8 shows a SDS-PAGE

gel with various stages of mouse GPI expression and purification. The expression of GST-GPI was found to be very good as more than estimated 25% of total bacterial protein being the recombinant protein The purity of the purified GPI protein was more than 95% as assessed by coomassie stained gels. The purified mouse GPI was used for epitope mapping studies and to establish mouse GPI ELISA and immunoblot screening assays.

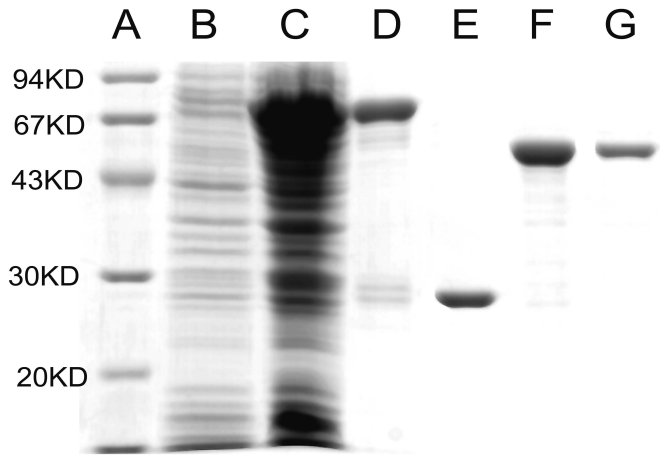

Figure 8. SDS-PAGE gel showing expression and purification of recombinant mouse GPI from BL21 cells. (**A**) Marker. (B) Un-induced bacterial lysate. (**C**) IPTG induced bacterial lysate. (**D**) Glutathione affinity purified GST-mGPI fusion protein. (**E**) GST alone. (**F, G**) Thrombin cleaved and purified mGPI.

4.1.2. Cloning and expression of truncated mouse glucose-6-phosphate isomerase.

In order to determine the epitopes recognized by the GPI-specific monoclonal antibodies by protein truncation assay, we cloned the following four truncated forms of mouse GPI protein into GST fusion vector pGEX-4T-3. i. GPI-I (GPI amino acid residues 1-190); ii. GPI-II (GPI amino acid residues 1-368); iii. GPI-III (GPI amino acid residues 324-559); iv. GPI-IV (GPI amino acid residues 485-559). The partial GPI clones were RT-PCR amplified from total C57BL6 thymus RNA using the following pairs of PCR primers respectively i. Forward-KN143 5´GGA GAA TTC TAT GGC TGC GCT CAC CCG GAA 3´ and Reverse-KN222 5´TTC CTC GAG TCG AGA GCT TCA GTC ACC ATG AG 3´; ii. Forward-KN143 5´GGA GAA TTC TAT GGC TGC GCT CAC CCG GAA 3´ and Reverse-KN224 5´TTC CTC GAG TCA GCA AAG CGG TGC ATG TAC TG 3´; iii. Forward-KN223 5´GGA GAA TTC TGG CAT CTG GTA CAT CAA CTG GC 3´ and Reverse-KN 144 5´TTC CTC GAG TCC CAA CAG CCT TGT AGT CC 3´; iv. Forward-KN 225 5´GGA GAA TTC TTT CAT TCT GGG GGC CTT GAT TG 3´ and Reverse-KN144 5´TTC CTC GAG TCC CAA CAG CCT TGT AGT CC 3´. The primers had EcoRI and XhoI recognition sites for in-frame cloning into pGEX-4T-3 vector. PCR was done with *Pfu* DNA polymerase for error free amplification. The PCR cycling parameters for GPI-I and GPI-IV were an initial denaturation at 95^{o}C for 2