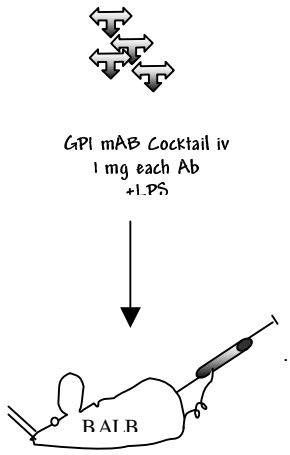
4.6. New glucose-6-phosphate isomerase specific antibody induced mice models for Rheumatoid Arthritis.

4.6.1. Establishment of glucose-6-phosphate isomerase specific monoclonal antibody transfer induced mice model for Rheumatoid Arthritis.

Mouse GPI specific monoclonal antibodies were successfully cloned. Epitope mapping studies showed that hybridoma clones 11H3.C10 and 1E3 secreted antibodies recognizing epitopes GPI 170-202 and the clone 46H9 secreted antibody recognizing epitope GPI 470-495. Injection of monoclonal antibodies to collagen-II into mice induced arthritis (Terato, Hasty et al. 1992) and on the similar lines it was attempted to establish a mice model with GPI antibodies. 2 mg of each purified GPI monoclonal antibodies were injected *i.v* either individually or as a mixture. in a volume of 200 *µl* saline into each mice. The next day 50 *ug* of LPS in saline was injected *i.p* to reduce the threshold for inflammation development. The mice were assessed for development of RA by measuring ankle thickness, clinical index and histology.

Day	Treatment
0	Antibody 2 mg each *i.v*
1	LPS 50 *ug i.p* / mice



GPI MAB INDUCED RA MODEL

Figure 45. Illustration of scheme of GPI monoclonal induced arthritis in mice.

The GPI reactive monoclonal antibodies 1E3, 11H3.C10 and 46H9 were pathogenic on transfer into naive mice. However only combination of antibody pairs recognizing different epitopes could induce disease(1E3 and 46H9 or 11H3.C10 and 46H9 or 1E3,11H3.C10 and 46H9) which means formation of GPI -immune complex is necessary for inducing RA.

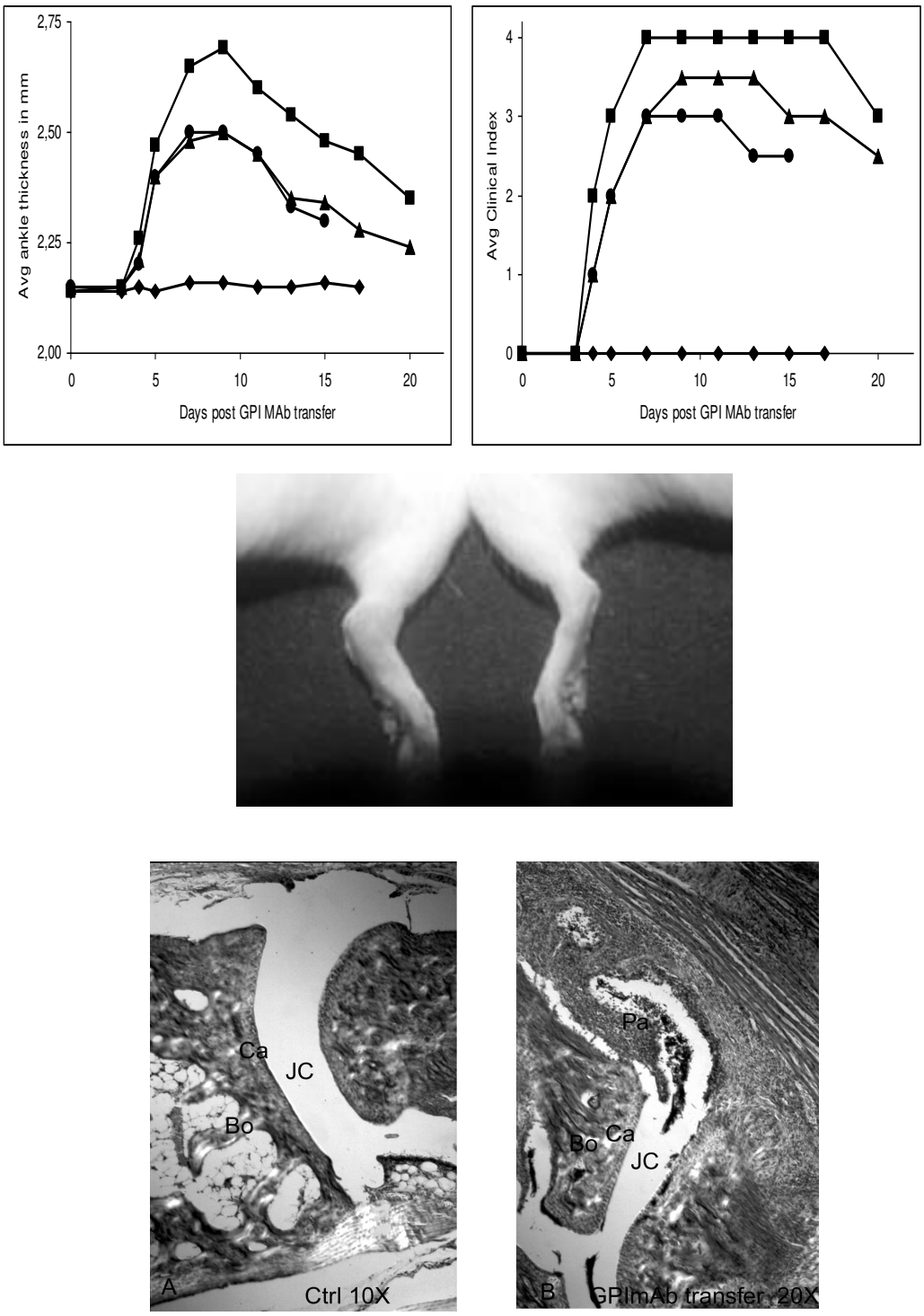
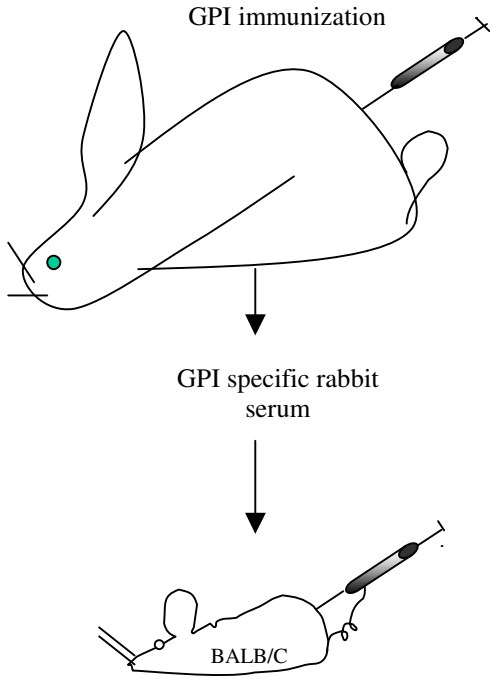


Figure 46. Joint inflammation in GPI monoclonal induced RA mice model. For GPI monoclonal induced arthritis 2.0 mg of each GPI mAb in a volume of 200 *µl* saline was injected *i.v* per mice. on day 0 followed injection of 50 µg LPS (*E.coli* strain 0111B4) *i.p.* on day 1. Average ankle thickness and clinical index(II) are shown. (■)1E3+11H3.C10+46H9 GPI mAb cocktail (▲)11H3.C10+46H9 mAb cocktail(●)1E3+46H9 mAb cocktail (♦) negative control. Joint histology of mice not treated and treated are shown. The GPI mAb treated mice shows inflamed joints with synovial inflammation. JC-joint cavity; Ca-cartilage; Bo-bone; Pa-pannus tissue.

4.6.2. Establishment of GPI anti-sera induced mice model for Rheumatoid Arthritis.

Pathogenic GPI-specific sera from the K/BxN RA mice was being used for inducing RA in naïve mice. In order to establish the fact, whether the pathogenicity of K/BxN antibodies is unique to the K/BxN mice or would be manifested in any antibody specific for GPI, rabbits were immunized with purified recombinant mouse GPI as shown in table below. The immunized rabbits were bled before and after immunizations and the reactivity sera against GPI was tested by western blot assay (figure 48). In order to test the arthritogenicity of the rabbit anti-mouse GPI antibodies the anti-sera was injected into BALB/c mice. The mice were injected *i.p* with 300 μl of either pre-immune rabbit sera or with 300 μl of immune sera tested positive for GPI on western blots.

Day	Immunization
0	200 *ug* mGPI in 500 μl saline + 500 μl CFA
14	200 *ug* mGPI in 500 μl saline + 500 μl IFA
21	200 *ug* mGPI in 500 μl saline + 500 μl IFA



Rabbit GPI sera transfer RA model

Figure 47. Illustration of scheme for generation of rabbit GPI sera induced RA model.

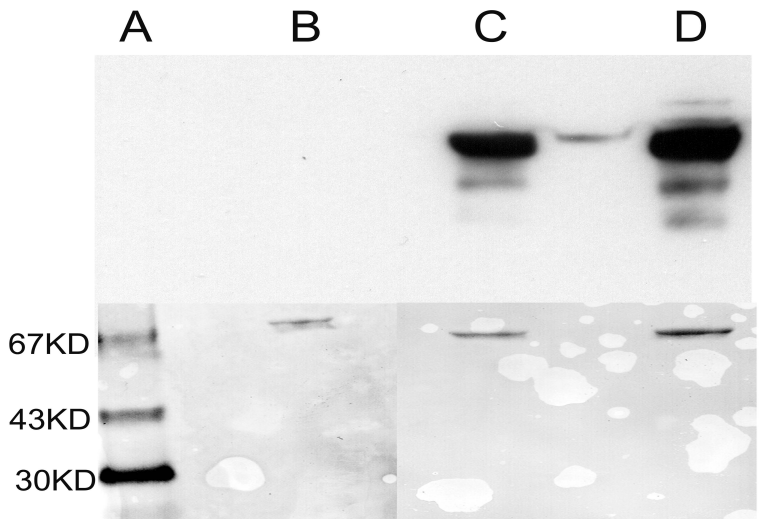


Figure 48. Reactivity of rabbit sera to recombinant GPI shown by western blotting. Western blot (upper) and Ponceau red stained blots(lower) showing the reactivity of sera from mouse GPI immunized Rabbit. (A)Marker. (B) Pre-immune rabbit sera. (C and D) mGPI immunized rabbit sera.

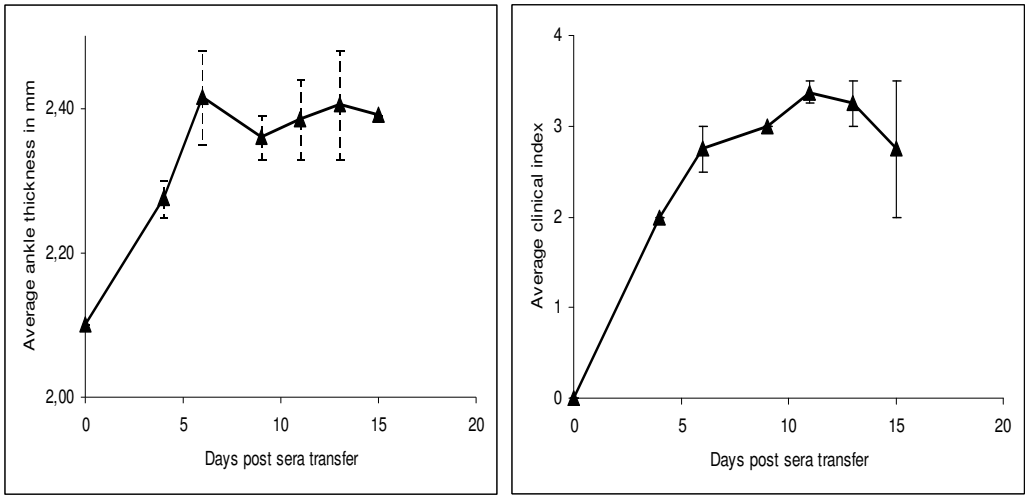


Figure 49. Ankle thickness and clinical index in rabbit anti mGPI sera induced RA in mice. Anti-sera to GPI raised in rabbits can induce RA in mice. 300 *µl* rabbit anti-mouse GPI sera was injected *i.p* per mice to induce arthritis. Average ankle thickness and clinical index(II) are shown. Data are expressed as mean ± SEM; n=3

The rabbit anti-GPI sera injected mice developed RA with increase in ankle swelling and increase in clinical index score. The inflammation kinetics was a bit delayed compared to K/BxN sera system but more prolonged with mice at day 15 still showing ankle swelling.

5.0. Discussions

5.1. Characterization of GPI reactive auto-antibodies from K/BxN mice.

The K/BxN murine arthritis model is very similar to many aspects to RA, including joint inflammation and eventual destruction of the synovial joints. Anti-GPI Abs alone on transfer into naive recipient mice is arthritogenic, highlighting the importance of anti-GPI immunoglobulins in the initiation phase of disease; thereby reviving a B-cell paradigm for RA pathogenesis (Benoist and Mathis 2000). Though recent efforts have been undertaken to study the antibody repertoires in K/BxN mice (Maccioni, Zeder-Lutz et al. 2002; Mandik-Nayak, Wipke et al. 2002), the pathogenic B cell epitopes still unknown. A major puzzle in the K/BxN model has been how antibodies to an ubiquitous antigen can lead to a joint specific disease. By using positron emission tomography, Wipke *et al* showed that purified anti-GPI IgG localize specifically to distal joints in the front and rear limbs within minutes of intravenous injection (Wipke, Wang et al. 2002) and Matsumoto et al by immunohistology showed that GPI is localized on surface of joints even in normal mice (Matsumoto, Maccioni et al. 2002). One possible reason, why the GPI antibodies are targeted to joints could be that anti-GPI Abs are cross-reactive to a joint-specific antigen (mimicry). Here the antibody specificity/cross reactivity would be responsible for pathogenicity. Another possible reason could that the GPI found in the joint is no different from that expressed in other organs and though autoantibodies to GPI, would bind to GPI in all organs and initiate the complement cascade, the absence of complement inhibitors on cartilage would result in full fledged inflammation only in joints. A completely different reason could be that the antibodies may inhibit/modulate the activity of secreted multifunctional GPI and this somehow could precipitate disease and in this case the altered GPI function may be the effector of the disease. Also often gylcosylation changes like lack of galactose on asparagine-linked oligosaccharides on the immunoglobulins has been associated with severity of RA (Rademacher, Williams et al. 1994) It was therefore important to elucidate the epitopes and post translational modifications of the pathogenic anti-GPI in K/BxN RA model. In this work, GPI reactive monoclonal antibodies were established from K/BxN mice spleen cells to investigate the pathogenicity and elucidate the epitopes on GPI. The efforts to clone these antibodies from spontaneously activated B cells from spleen of 1 year old arthritic mice resulted only in GPI reactive clones of the IgM isotype and no IgG clones. The IgM clones were also found to be cross reactive to other proteins. Reports have been published of similar generation of GPI reactive monoclonal antibodies from naïve K/BxN mice (Maccioni, Zeder-Lutz et al. 2002). The reason for their success in cloning IgG type antibodies could be due to use of young 29-

60 day old mice. The problem of not being able to clone GPI reactive IgG_1 antibodies was overcome by boosting K/BxN mice with GPI, to activate GPI reactive B-cells before fusion. Both the hybridoma fusion experiments unusually high number of GPI reactive clones were obtained reflecting the very strong T and B cell collaboration in K/BxN mice. Three stable GPI reactive IgG_1 monoclonal antibody secreting clones were chosen for antibody purification and analysis. Two of these clones 11H3.C10 and 46H9 had been generated from a 6 month old male K/BxN while clone 1E3 was from 6 month female K/BxN mice. The purified monoclonal antibodies recognized the SDS denatured and reduced mouse GPI on western blots, which meant these antibodies recognized linear epitopes on GPI. A simple and novel technique peptide fingerprint western blotting was used to differentiate clones based on epitope recognition. Using this technique it could be identified that mAbs 11H3.C10, 1E3 recognized the same epitope on GPI (even though they have been generated from two different mice), while mAb 46H9 recognized a different epitope. Also comparing western blot band the intensities and patterns between the mAbs and KBN sera, comments on relative ratio/titers of the respective Abs in KBN sera could be made. The K/BxN sera are composed of atleast two major dominant GPI epitope binding antibodies (1E3/11H3.C10 and 46H9 epitope). Of these 11H3.C10/1E3 epitope binding antibodies comprise the dominant antibody population in K/BxN sera when compared to the 46H9 epitope variety. Mass spectrometric epitope mapping technique was used to identify epitopes of the GPI antibodies. Fine epitope mapping could be achieved by this technique with microgram quantities of antibodies and in a short time compared to conventional mapping methods. The epitopes of GPI mAb 11H3.C10 and 1E3 were mapped to GPI peptide residues 170-202, while for the mAb 46H9 epitopes was mapped to GPI peptide residues 470-495. A parallel study by GPI protein truncation and western blotting confirmed once again the mass spectrometrically identified epitopes. Modeling of the epitopes on crystal structure of rabbit GPI showed that the epitope regions are located on surface of the GPI homodimer molecule and very interestingly the epitopes of 11H3.C10/1E3 was located at the GPI dimer interface. Even though the epitopes do not comprise the active site residues of GPI, it presence close to the site could be inhibitory for GPI activity due to steric hindrance effects from the bound antibody. Studies by Schaller *et al*. have suggested that GPI might be the auto-antigen in human RA as well. They also claimed that GPI could be used to diagnose 64% of the RA cases (Schaller, Burton et al. 2001). A parallel analysis of human sera from patients with RA was carried out using recombinant HPLC-purified human GPI cloned and expressed in this work. GPI reactivity could be detected only in only a few serum samples from RA as well (Kassahn, Kolb et al. 2002). We

report that GPI as a auto-antigen in human RA but not as high as in 64% of patients claimed above. Efforts are now underway to map epitopes of GPI antibodies from RA patients.

5.2. Role of Innate immunity mediators

5.2.1. K/BxN sera induced arthritis is dependent on the alternative complement pathway activation and no role for classical complement pathway.

Inflammation typically seen in arthritis, glomerulonephritis and vascultis involves formation and deposition of soluble immune complexes. The activation of the classical complement pathway is believed to be the major effector mechanism in a response involving immune complexes, while the activation of alternative complement pathway is normally involved in inflammatory responses due to microbial products and IgA. IgG lacking terminal galactose in the terminal GlcNAc residues have been known to bind mannose-binding lectin and activate the a third complement pathway, the lectin pathway (Malhotra, Wormald et al. 1995). Surprisingly here it could be shown using the $C4^{-/-}$ mice that the activation of classical and the lectin complement pathways and the split complement product C4b do not have a significant role in mediating inflammation in the K/BxN sera transfer RA mice model. While lack of inflammation seen in NOD mice, coupled with the observation of a drastic decrease in inflammation seen in cobra venom factor treated, complement depleted mice showed that the alternative complement activation pathway has an important role in mediating inflammation in K/BxN sera transfer induced RA (Solomon, Kolb et al. 2002). The involvement of K/BxN antibodies in activation of the alternative complement pathway was not expected, as common (textbook) knowledge proposes antibodies to be mainly activators of classical complement pathway. A possibility that pathogenic GPI antibodies, due to abnormal glycosylation activating the lectin complement pathway (Malhotra, Wormald et al. 1995) can be ruled out here from the data from $C4^{-/-}$ mice. Also in line with the results seen here, reports have been published previously, which show that $C3^{-/-}$ and Factor $B^{-/-}$ mice were protected from CIA (Heitalla 2002), and also 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). In K/BxN sera transfer induced RA it was shown that mice lacking alternative complement components C3 and factor B are protected from inflammation (Ji, Ohmura et al. 2002). It was observed that NOD mice and complement depleted mice are resistant to K/BxN sera induced RA. Recently the disease locus responsible for resistance to RA seen in NOD mice, in the K/BxN [Ji, 2001 #1 and in CIA mice model (Johansson, Sundler et al. 2001) was exclusively mapped to the C5 and the FcγRIIb locus was shown not

be involved. Complement C3, C5a and C5aR receptor play a crucial role in mediating inflammation in a spectrum of autoimmune disease including arthus reaction (Baumann, Kohl et al. 2000; Baumann 2001; Bhatia, Saluja et al. 2001) and experimental allergic asthma (Karp et al, 2000). The cleavage of C5 by the alternative pathway C5 convertases may be an important effector in pathogenesis of K/BxN sera induced RA. Rightly so complement activation pathway, particularly the C5 and C5aR have become increasingly the favorite targets to inhibit inflammation recently (Wang, Rollins et al. 1995; Makrides 1998).

5.2.2. No role for complement receptors CR1 and CR2 in K/BxN sera induced RA.

CR1 is known to have an important role in immune complex clearance, inhibition of C3 and C5 convertase and as receptor for C3b/C4b (Krych-Goldberg and Atkinson 2001). On phagocytic cells CR1 mediates adherence and ingestion of C3b/C4b coated particles and also mediates, the transport and clearance of immune complexes. The lack of protection in *Cr2*$^{-/-}$ mice suggests that the absence of complement receptors CR1 had no significant affect on outcome of K/BxN sera induced RA (Solomon, Kolb et al. 2002). Also it could be shown that the *Cr2*$^{-/-}$ mice have no impaired clearance of GPI antibodies. This is in contrast to observations that a reduction in CR1 expression is associated with the deposition of ICs, in glomerulonephritis and SLE (Gatenby 1991). The rapid clearance of GPI antibodies from peripheral blood of the highly susceptible BALB/c mice, in contrast to RA resistant NOD mice could be interpreted as enhanced deposition of the antibodies at the site of inflammation due to complement activation. The complement receptor CR2 acts as a receptor for complement product C3dg, involved in germinal center reaction and is an activator of alternative pathway on binding iC3b (Schwendinger, Spruth et al. 1997). The observation of the absence of protection seen in *Cr2*$^{-/-}$, thus argues for a lack of role for CR2 in the context of above mentioned functions in K/BxN sera induced RA pathogenesis.

5.2.3. Activation of FcγRIII receptors mediates inflammation in K/BxN sera induced RA.

In order to analyze the influence of FcR family members in the development of arthritis, K/BxN sera was injected in FcγRIII and FcγRIIb deficient mice. The binding of FcγR receptors initiates signaling cascades that can lead to either activation (FcγRI and FcγRIII) or deactivation (FcγRII) of effector cells. It could be shown here that the *FcγRIII*$^{-/-}$ mice were completely resistant to RA on transfer of 100 *ul* K/BxN sera, while contrastingly the *FcγRIIb*$^{-/-}$ mice were highly susceptible, showing an accelerated onset and severe arthritis. The above

observations are agreeing to the known function of FcγRs.In *FcγRIII*$^{-/-}$ mice the absence of FcγRIII results in lack of disease as the binding of these receptors can activate effector cells and in *FcγRIIb*$^{-/-}$ mice the absence of FcγRIIb results in enhancement of disease as the binding of these receptors initiates signaling cascades that leads to deactivation of effector cells. The FcγR knockout mice lacking both the FcγRI and FcγRIII were found to be resistant to K/BxN sera induced arthritis (Kyburz, Carson et al. 2000). Also Ji *et al.* using the *FcγRIII*$^{-/-}$ mice could show that the K/BxN sera induced arthritis is dependent on the activation of the FcγRIII by the pathogenic antibodies in the effector phase of disease (Ji, Ohmura et al. 2002). Initial reports have attributed the effectors of inflammation to either FcR or to complement activation. But recently many reports argue for a co-dominant role of the both the systems (Heller, Gessner et al. 1999; Kohl and Gessner 1999; Baumann, Kohl et al. 2000). *FcγRIII*$^{-/-}$ mice are highly protected from IgG-induced hemolytic anemia, CIA (Stahl, Andren et al. 2002) and show impaired Arthus reaction (Baumann, Kohl et al. 2000), suggesting a dominant role of FcγRIII. Similarly from the results here, for the K/BxN sera induced mice model the important co-dominant role of both complement and FcγRs could be demonstrated.

5.2.4. Inhibition of mast cells degranulation and administration of histamine H1 receptors antagonists ameliorates K/BxN sera induced RA.

Mast cells play a key role in RA inflammation. By administration of mast cell degranulation inhibitors cromolyn and tranilast and histamine receptor antagonists mepyramine and cimetidine the role of mast cell granulation and role the of histamine action through the histamine receptor in K/BxN sera induced RA has been shown. 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. Mast cells are known to be involved in producing the first wave of TNFα secretion recruiting neutrophils to sites of inflammation (von Stebut, Metz et al. 2002). Synovial mast cells up regulate the C5aR in inflammation (Kiener, Baghestanian et al. 1998) and activation of mast cells by C5a results in inflammatory cytokine secretion (Woolley and Tetlow 2000). Recently the *W/Wv* and *Sl/Sld* mice strains lacking mast cells were shown to be completely resistant to K/BxN sera induced RA (Lee, Friend et al. 2002). Here again it could be shown that mice treated with degranulation inhibitors, cromolyn and tranilast developed reduced disease due to impaired mast cell degranulation. Also here the importance of histamine, a important vaso-active amine mediator released from mast cell granules on activation could be demonstrated**.** Histamine has diverse functions including local dilation of small vessels, increased vascular

permeability and gastric acid secretion. Blocking of histamine receptors in zymosan induced peritonitis in mice resulted in decreased plasma exudation, leukocyte influx, MCP-1 and IL-1β production (Kolaczkowska 2001). The results with histamine receptor antagonists points out the important role of histamine in K/BxN sera induced RA as well and are in line with the known function of these receptors. Histamine acts through three receptors H1, H2 and H3 receptors present on mast cells, lymphocytes and endothelium. Histamine H1 receptor is mainly involved in mediating inflammation (Baroody and Naclerio 2000) by enhancing vascular permeability, while H2 histamine receptor mainly mediates gastric acid secretion (Del Valle and Gantz 1997) and H3 histamine receptor involved in behavioral responses. It was observed that administration of H1 histamine receptor antagonist mepyramine significantly reduced ankle thickness and clinical index (69% and 54% respectively), while the H2 histamine receptor antagonists cimetidine administration did not have a comparable effect (only 14.5% and 0.0% respectively).

5.2.5. Administration of chemokine receptor CXCR2 antagonists ameliorates K/BxN sera induced RA.

Investigations into the role of the CXC chemokine, MIP-2 and its receptors CXCR2 in neutrophil recruitment and trafficking in early inflammatory response to transferred K/BxN arthritogenic serum were studied. Previously it had been shown that in the absence of neutrophils, mice were completely resistant to the inflammatory effects of K/BxN serum. Whereas *gp91^{phox}*-deficient mice and *iNOS2* knockout mice which are unable to generate NO, and hydrogen peroxide respectively developed arthritis with similar kinetics (Wipke and Allen 2001). Recent evidence suggests that neutrophils are recruited by mast cells to sites of inflammation by producing TNFα and MIP-2 (Biedermann, Kneilling et al. 2000; von Stebut, Metz et al. 2002). IL8-R knockout mice are defective for neutrophil migration (White 1998; Godaly, Hang et al. 2000). Blockade of IL-8 or IL8 receptor has been shown to reduce inflammation (Miura, Fu et al. 2001) suggesting that these molecules are likely to be important in the K/BxN model for neutrophil recruitment. MIP-2 (IL-8 in humans) activates neutrophils by binding to two distinct G-protein coupled receptors CXCR1 and CXCR2. Other CXC chemokines like GROα, GROβ, GROγ, and ENA-78 bind and activate only CXCR2. We treated BALB/c mice with compound SB 225002, a potent, selective non-peptide CXCR2 antagonist to study the role of CXCR2 receptor in neutrophil recruitment. The mice treated with CXCR2 antagonists showed a strong reduction in ankle thickness and clinical index (80% and 75% respectively), arguing for a important role for the CXCR2

receptor in K/BxN sera induced RA. However surprisingly the administration of blocking antibodies to the CXC chemokine MIP-2 in mice transferred with K/BxN sera did not significantly reduce arthritis, compared to that seen in blocking its CXCR2 receptor. Though this may be due to lack of proper dosing of the MIP-2 antibodies, it could be also possible that presence of other related ligands of CXCR2 can substitute the function of MIP-2 here. Moreover reports have been made in collagen-II mAb induced RA mice model wherein also administration of MIP-2 blocking antibodies did not reduce inflammation (Kagari, Doi et al. 2002).

5.2.6. Macrophages depleted mice are resistant to K/BxN sera induced RA.

Macrophages are present in high numbers in inflamed tissues especially at the cartilage-pannus interface in RA and correlate with severity of disease. 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. It could be shown here that macrophages have a key role in K/BxN sera induced RA, as BALB/c mice systemically depleted of macrophages by clodronate liposome treatment were completely resistant to K/BxN sera induced RA. Macrophages become the third key innate cellular player implicated in K/BxN sera induced arthritis, after neutrophils (Wipke and Allen 2001) and mast cells (Lee, Friend et al. 2002). MCP-1, MIP-1a/β and RANTES are chemoattractant to monocytes/macrophages (Yuan, Masuko-Hongo et al. 2001). Recent studies have shown that monocytes are recruited to sites of inflammation through CCR2 receptors by MCP-1 and after differentiation into macrophages and up regulation of CCR1 and CCR5, leads to predominant recruitment by MIP-1α (Kaufmann, Salentin et al. 2001). Antagonist to MCP-1 inhibited arthritis in MRL-lpr mice model (Gong, Ratkay et al. 1997). However the studies done here with the blocking of MCP-1 with antibodies did not show any significant reduction inflammation in the K/BxN sera induced RA model.

5.2.7. TNFα has a dual role in K/BxN sera induced RA.

Tumor Necrosis Factor α plays a pivotal role in the cytokine cascade that results in joint inflammation and destruction in RA. It could be observed that administration of TNFα neutralizing ameliorates RA in BALB/c mice transferred with K/BxN sera. The mice showed a 32.2 % reduction in ankle thickness and an even more drastic 75.0 % reduction in clinical index. This meant that TNFα inhibition reduced incidence of RA (number of ankles affected) but increased the swelling of the affected limbs. This could be partially explained due to

inhibition of preformed TNFα released from degranulation mast cells. This TNFα is responsible for recruitment neutrophils in the inflammation cascade (Chen 2001; Stebut 2002). Contrastingly, however it was observed that TNFR1 and TNFR2 mice deficient mice both developed severe RA on transfer of K/BxN sera. In fact the *TNFR*$^{-/-}$ mice, especially the *TNFR2*$^{-/-}$ had more severe RA compared to C57BL/6 mice controls. This observation was quite unexpected, as TNFR1 and TNFR2 are the only known receptors for TNFα. There could be several reasons for the strong arthritis that develops in TNFR1/2-deficient mice. The most straightforward explanation is that other receptors can compensate and mediate TNFα signals. The second reason could be lack of apoptosis of activated cells at site of inflammation due to absence of apoptosis inducing TNFR. The dual effect of the blockade of TNFα and absence of its receptor TNFR1 and TNFR2 on the outcome of disease seen here generally reflects the incomplete effectiveness of TNFα therapy seen in RA patients. Though TNFα and TNFR treatment has been successful in reducing joint inflammation in RA, still about 30% of the patients do not respond to the therapy. Previously also, in mice conflicting roles for TNFα mice has been observed. RA is known to proceed in the absence of TNFα (Campbell, O'Donnell et al. 2001) or TNFR (Mori, Iselin et al. 1996). In collagen-II mAb induced RA mice model administration of neutralizing TNFα antibodies also protected from RA (Kagari, Doi et al. 2002). A recent study using K/BxN sera transfer model reported that the TNFα deficient mice developed no disease upon transfer of K/BxN serum, either clinically or histologically. However strangely a substantial number of animals did develop joint inflammation (9/23). Also *TNFR1*$^{-/-}$ and *TNFR2*$^{-/-}$ mice developed full fledged disease on K/BxN sera transfer (Ji, Pettit et al. 2002).

5.3. New animal models in RA.

An anti-GPI monoclonal antibody induced RA model on similar lines to collagen-II monoclonal antibody induced RA in mice (Terato, Hasty et al. 1992) and an anti-GPI sera induced RA model in BALB/c mice could be successfully established. The GPI reactive monoclonal antibodies could transfer disease in pairs that bind to separate epitopes on GPI (11H3.C10 and 46H9 or 1E3 and 46H9), cleary suggesting that GPI immune complexes are involved in pathogenesis in K/BxN model. The antibodies on transfer either form pathogenic immune complexes on the cartilage surface as an array or deposited on cartilage after forming in the circulation The GPI immune complex deposited on joints are then capable of activating the alternative complement pathway and FcγRs on cells in the K/BxN model It can be assumed that the pathogenicity of these GPI specific K/BxN antibodies may be depend on its

post translational modifications like abnormal glycosylation or can simply depend on its antigen specificity or both. Another point to be noted is that K/BxN antibodies are unique in activating the alternative complement pathway (Ji et al, 2002, Solomon et al, 2002). If the pathogenicity of the antibodies was due its post translational than anti-GPI specific sera from mouse GPI immunized animals like raabits would not substitute for K/BxN mice antibodies in inducing RA in mice as it would be unlikely these antibodies would have similar post translational decorations. However on the contrary, it could be showed here, that on transfer of the anti mouse GPI rabbit antibodies into naive BALB/c mice, the mice was found to develop full fledged disease similar to K/BxN sera induced RA. This clearly rules out the role of K/BxN GPI-antibody specific postaranslational modification in mediating pathogenicity in K/BxN model. The GPI sepecificity of the antibodies alone in both cases seems good enough to have a pathogenic effector role by targeting the antibodies to joints to form GPI immune complexes. The proinflammatory environment of joints space coupled with absence of complement activation inhibitor molecules like membrane cofactor protein and decay accelerating factor on cartilage, leads to activation of alternative complement pathway, and thus inflammation leading to arthritis. Surprisingly the immunized rabbits did not show signs of joint inflammation even though they had high titres of GPI antibodies after immunization. It would to be interesting to analyze why only the mice develop disease and not the rabbits, even though rabbit and mouse GPI have more than 90% sequence homology and hence could cross react. Also a possibility why rabbits did not deveop RA could be that rabbit unlike mice may not have GPI deposited on joint cartilage to bind the GPI antibodies. Further studies involving, comparison of the GPI monoclonal induced RA model, GPI anti-sera induced mice RA model and the elucidation of reason for RA resistance seen in rabbits would help throw more light in understanding the role of GPI specific autoantibodies in RA.

6.0. Summary

K/BxN is a most recent Rheumatoid Arthritis murine model, where on crossing the KRN-TCR transgenic mice with NOD mice, the F1 K/BxN off-spring's develop spontaneous arthritis at about 3rd week after birth. The antigen recognized by the KRN TCR in the context of MHC-II I-A^{g7} as well as the arthritogenic immunoglobulin was identified as glucose-6-phosphate isomerase (GPI)—a glycolytic enzyme that is expressed by all cells (Korganow et al. 1999; Matsumoto et al. 1999). Transferring just 100 μl of KBN sera into healthy as well as in lymphocyte deficient mice, could induced RA. Interestingly it could be demonstrated that also RA patients have autoantibodies to GPI using recombinant human GPI ELISA assays (Kassahn et al. 2002). To elucidate the reason for the pathogenicity and the epitopes of anti-GPI antibodies, recombinant mouse GPI was expressed and GPI-specific monoclonal antibodies were generated from the K/BxN mouse. The epitope of the mAbs were mapped by a combination of peptide fingerprinting western blot, high-resolution mass spectrometry and protein truncation studies. Transfer of GPI mAb pairs, which bind to different epitopes, could induce arthritis in naive mice. Knockout mice as well *in vivo* blocking/inhibition studies were used to elucidate the role of innate immune mediators in K/BxN sera induced RA. Tables A and B summarizes the knockout phenotypes analyzed and in vivo inhibition studies done to date from this work and others on this system. Using the $C4^{-/-}$ mice, NOD mice and complement depletion study, it could be shown that the K/BxN antibodies do not activate the classical, but the alternative complement pathway to mediate RA (Solomon et al 2002). Studies from $Cr2^{-/-}$ mice showed that the complement receptors 1 and 2 have no modulatory role in K/BxN sera induced RA (Solomon et al. 2002). On analysis of role of Fc receptors in K/BxN sera transfer induced RA, it was found that the $Fc\gamma RIIb^{-/-}$ mice were highly susceptible whereas $Fc\gamma RIII^{-/-}$ mice were completely resistant to disease. Also it could be shown that the $TNFR1^{-/-}$ and $TNFR2^{-/-}$ mice both developed severe disease on K/BxN sera transfer whereas blocking TNFα with anti-TNFα antibodies ameliorated RA, hence supporting a dual role of TNFα in RA. The mast cell degranulation and H1 histamine receptor inhibition *in vivo* significantly reduced inflammation in K/BxN sera induced RA, in this study. Also *in vivo* inhibition of the CXCR2 receptor led to significant reduction in RA, thus reflecting its important role in neutrophil recruitment. The *in vivo* depletion of macrophages in mice, led to complete resistance to inflammation in K/BxN sera induced RA, pointing to a key role for this cell in RA pathogenesis. In conclusion the important role for autoantibodies and innate immunity mediators in RA pathogenesis has been demonstrated from the studies in the K/BxN murine model for RA.

Table A. Gene knockout mice studies in K/BxN sera induced model.

Disrupted gene	Targets	Disease outcome	References
Rag-1	T and B cells	susceptible	(Korganow et al. 1999)
W/Wv and Sl/Sld	Mast cells	resistant	(Lee et al. 2002)
C1q and C4	Classical and Lectin complement activation	susceptible	(Ji et al, 2002, Solomon et al, 2002)
C3	Complement activation	resistant	(Ji et al. 2002)
C5 and C5aR	Complement activation	resistant	(Ji et al. 2002)
Factor B	Alternative complement activation	resistant	(Ji et al. 2002)
MBP-A	Lectin complement pathway	susceptible	(Ji et al. 2002)
C6	Membrane attack complex	susceptible	(Ji et al. 2002)
Cr2	Complement receptor 1/2	susceptible	(Ji et al, 2002, Solomon et al, 2002)
Fcγ R	FcγRI/III signaling	resistant	(Kyburz et al. 2000)
Fcγ RI	FcγRI signaling	susceptible	(Ji et al. 2002)
Fcγ RIIb	FcγRIIb signaling	susceptible	(Ji et al. 2002) and *
Fcγ RIII	FcγRIII signaling	weak disease/resistant	(Ji et al. 2002) and *
IL-1R	IL-1 R signaling	resistant	(Ji et al. 2002)
IL-6	IL-6	susceptible	(Ji et al. 2002)
TNFα	TNF-α	susceptible	(Ji et al. 2002)
Ltα	Ltα	susceptible	(Ji et al. 2002)
TNFR1/2	TNFR1/2 signaling	delayed disease	(Ji et al. 2002)
TNFR1	TNFR1 signaling	susceptible	(Ji et al. 2002) and *
TNFR2	TNFR2 signaling	susceptible	(Ji et al. 2002) and *
TRANCE/RANKL	TRANCE/RANKL signaling	no bone erosion	(Pettit, et al 2001)
iNOS2	NO reactive species	susceptible	(Wipke and Allen 2001)
gp91 (phox)	Oxygen reactive species	susceptible	(Wipke and Allen 2001)
CD40L	CD40L signaling	resistant	(Kyburz et al. 2000)

Table B. *In vivo* therapy/ antibody blocking studies in K/BxN sera induced model.

Therapy	Targets	Disease outcome	References
RB6-8C5	Neutrophils	resistant	(Wipke and Allen 2001)
SB225002	CXCR2 receptor	reduction of 80% in AT & 75% in CI	*
MIP-2 Ab	blocks MIP-2	reduction of 11.9% in AT & 12.5% in CI	*
Clodronate	Macrophages	resistant	*
MCP-1	blocks MCP-1	reduction of 5% in AT & 12.5% in CI	*
Cobra Venom Factor	complement	weak disease	*
C5 mAb	blocks C5	resistant	(Ji, Ohmura et al. 2002)
TNFα Ab	blocks TNFα	reduction of 32 % in AT & 75% in CI	*
Cromolyn	Mast cell degranulation	reduction of 63.6 % in AT & 56.6% in CI	*
Tranilast	Mast cell degranulation	reduction of 41.8 % in AT & 16.75 % in CI	*
Mepyramine	H1 histamine receptor	reduction of 69% in AT & 54% in CI	*
Cimetidine	H2 histamine receptor	reduction of 14.5% in AT & 0% in CI	*

AT-ankle thickness, CI-clinical index score.

**Contributed from this thesis work.*

7.0. Zusammenfassung

K/BxN ist ein neues Mäusmodell der rheumatoiden Arthritis, bei dem sich ab der 3 Woche nach der Geburt in der F1 Generation der Kreuzung von KRN-TCR transgenen Mäusen mit NOD-Mäusen spontan Arthritis entwickelt. In den K/BxN Mäusen wurde das Antigen, das durch den KRN-TCR im Kontext mit dem MHC-II I-A^{g7} und durch arthritogene Antikörper erkannt wird, als Glukose-6-Phosphate Isomerase (GPI) definiert. GPI ist ein glykolytisches Enzym, das in allen Zellen exprimiert wird (Korganow et al. 1999; Matsumoto et al. 1999). Die Übertragung von 100 *µl* der KBN-Seren aus erkrankten in gesunde Tiere verursacht Arthritis. Dies geschiet auch in Lymphozyten defizientenTieren. Interessanterweise konnte mit Hilfe des humanen rekombinanten GPI und eines GPI ELISAs gezeigt werden, dass auch RA-Patienten Autoantikörper gegen GPI bilden (Kassahn et al. 2002). Um die Ursache der Pathogenität und die Identität der Epitope der anti-GPI Antikörper aufzuklären, wurde recombinantes murines GPI hergestellt und GPI-spezifische monoklonale Antikörper aus erkrankten K/BxN Mäusen gewonnen. Die Definition der Antikörper-Epitope erfolgte durch Western Blot Analyse mit partiell verdauter GPI, sowie mittels Massenspektrometrie. Die simultane Injektion dieser monoklonalen Antikörper, die an unterschiedliche Epitopes binden, konnte Arthritis in naiven Mäusen auslösen. In vivo Experimente mit verschiedenen Inhibitoren und verschiedenen Knockout-Mäusestämmen wurden durchgefürt, um die Rolle des angeborenen Immunsystems als Vermittler im K/BxN Modell aufzuklären. Tabellen A und B fassen die Ergebnisse mit den Inhibitoren und Knockout-Mäusestämmen zusammen, die in dieser Arbeit und von anderen beschrieben wurden. Bei Untersuchungen mit *C4* $^{-/-}$ Mäusen, NOD-Mäusen und Complement-Depletionsversuchen konnte gezeigt werden, dass die K/BxN Antikörper nicht den klassischen, sondern den alternativen Komplementpfad aktivieren (Solomon et al 2002). Untersuchungen an *Cr2* $^{-/-}$ Mäusen zeigte dass weder CR1 noch CR2 eine essentielle Rolle bei der Arthritis im K/BxN Modell spielen (Solomon et al. 2002). Bei der Analyse der Rolle von Fc rezepteron stellte sich heraus, dass *FcγRIIb*$^{-/-}$ Mäuse schwer krank werden, während *FcγRIII*$^{-/-}$ Mäuse vollständig geschützt sind. Darüber hinaus konnte gezeigt werden, dass *TNFR1* $^{-/-}$ und *TNFR2* $^{-/-}$ Mäuse verstärkt Arthritis nach K/BxN Serum Transfer entwickeln, während TNFα blockierende Antikörper Arthritis verringern. Auch ist die Arthritis nach *in vivo* Hemmung von Mastzelldegranulierung und die Blockierung des Histamin1 Rezeptors stark reduziert. Die Hemmung des CXCR2 Rezeptors führte zu einer starken Verringerung der Arthritis, was auf eine wichtige Rolle dieses Rezeptors bei der Einwanderung von Neutrophilen schliessen lässt. Die entfernung von Makrophagen in vivo führtezur Verhinderung der Arthritis, womit eine weitere essentielle

Zellart definiert ist. Damit konnte die withtige Rolle von Auto-antikörper und Mediatoren des angeborenen Immunsystems in der Pathogenese der RA in K/Bx-Maus modell nachgewiesen werden.

Tabelle A. Die genetische Knockoutmaus-Studie im K-/BxN- Serum induzierten Modell.

Betroffene Gene	**Ziele**	**Krankheitsbild**	**Referenzen**
Rag-1	T und B Zellen	anfällig	(Korganowet al. 1999)
W/Wv und Sl/Sld	Mastzellen	resistent	(Lee et al. 2002)
C1q and C4	Komplementaktivierung über den klassischen und den Lektin pfad	anfällig	(Ji et al, 2002, Solomon et al, 2002)
C3	Komplementaktivierung	resistent	(Ji et al. 2002)
C5 and C5aR	Komplementaktivierung	resistent	(Ji et al. 2002)
Faktor B	Alternative Komplementaktivierung	resistent	(Ji et al. 2002)
MBP-A	Komplementaktivierung über den Lektinpfad	anfällig	(Ji et al. 2002)
C6	Membranangreifender Komplex	anfällig	(Ji et al. 2002)
Cr2	Komplementrezeptor 1/2	anfällig	(Ji et al, 2002, Solomon et al, 2002)
FcγR	FcγRI/III Signal	resistent	(Kyburz et al. 2000)
FcγRI	FcγRI Signal	anfällig	(Ji et al. 2002)
FcγRIIb	FcγRIIb Signal	anfällig	(Ji et al. 2002) and *
FcγRIII	FcγRIII Signal	leicht krank /resistent	(Ji et al. 2002) and *
IL-1R	IL-1 R Signal	resistent	(Ji et al. 2002)
IL-6	IL-6	anfällig	(Ji et al. 2002)
TNFα	TNF-α	anfällig	(Ji et al. 2002)
Ltα	Ltα	anfällig	(Ji et al. 2002)
TNFR1/2	TNFR1/2 Signal	verzögertes Krankheitsbild	(Ji et al. 2002)
TNFR1	TNFR1 Signal	anfällig	(Ji et al. 2002) and *
TNFR2	TNFR2 Signal	anfällig	(Ji et al. 2002) and *
TRANCE/RANKL	TRANCE/RANKL Signal	keine Knochenerosion	(Ji et al. 2002)
iNOS2	NO reaktive Spezies	anfällig	(Wipke and Allen 2001)
gp91 (phox)	O reaktive Spezies	anfällig	(Wipke and Allen 2001)
CD40L	CD40L Signal	resistent	(Kyburz et al. 2000)

Tabelle B. In vivo Therapie /Antikörper-Studien im K-/BxN-Serum induzierten Modell.

Therapie	**Ziele**	**Krankheitsbild**	**Referenzen**
RB6-8C5	Neutrophile	Resistent	(Wipke and Allen 2001)
SB225002	CXCR2 Rezeptor	Abnahme von AT um 80% und CI um 75%	*
MIP-2 Ab	blockiert MIP-2	Abnahme von AT um 11.9% und CI um12.5%	*
Clodronate	Makrophagen	Resistent	*
MCP-1	blockiert MCP-1	Abnahme von AT um 5% und CI um 12,5%	*
Cobra Venom Faktor	Komplement	schwache Krankheit	*
C5 mAb	blockiert C5	Resistent	(Ji, Ohmura et al. 2002)
TNFα Ab	blockiert TNFα	Abnahme von AT um 32% und CI um 75%	*
Cromolyn	Mastzellen Degranulierung	Abnahme von AT um 63,6% und CI um 56,2%.	*
Tranilast	Mastzellen Degranulierung	Abnahme von AT um 41,8% und CI um 16,75%.	*
Mepyramine	H1 Histaminrezeptor	Abnahme von AT um 69% und CI um 54%	*
Cimetidine	H2 Histaminrezeptor	Abnahme von AT um 14,5% und CI um 0%	*

AT-ankle thickness, CI-clinical index score.

* Beitrag von dieser Doktorarbeit.

8.0. References.

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