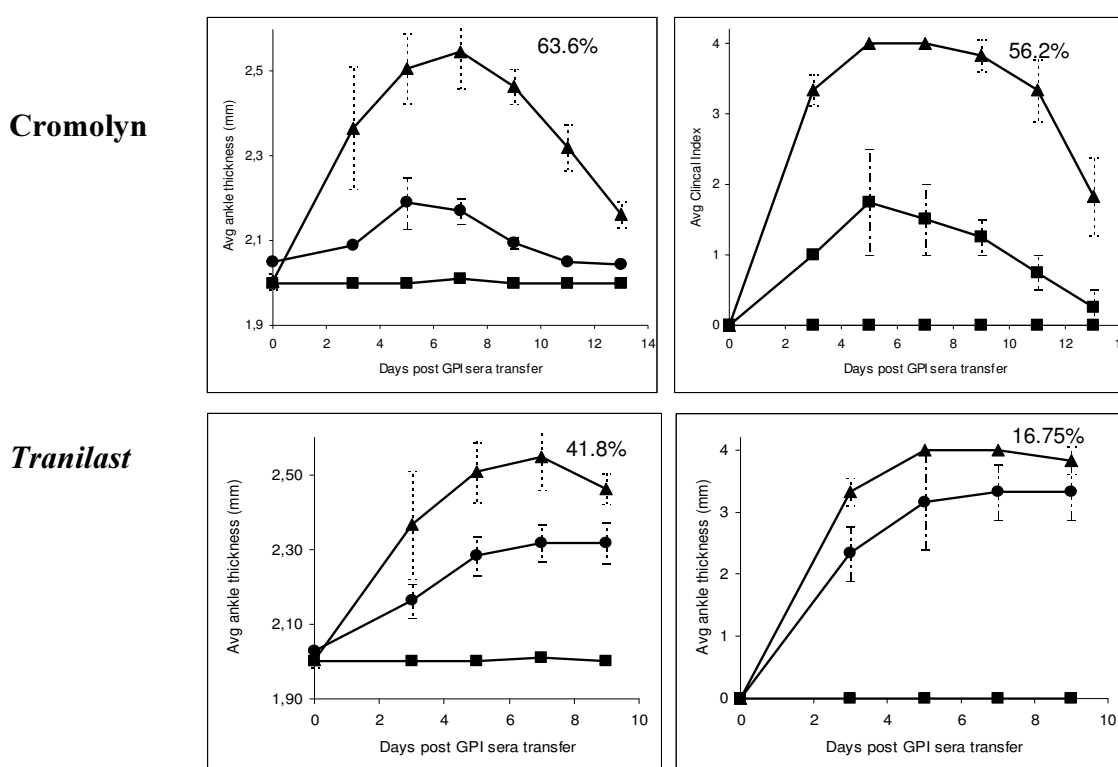


#### 4.4. Role of innate immunity cellular players in RA.

##### 4.4.1. Mast cell de-granulation inhibitors and Histamine H1 receptor antagonist ameliorates RA.

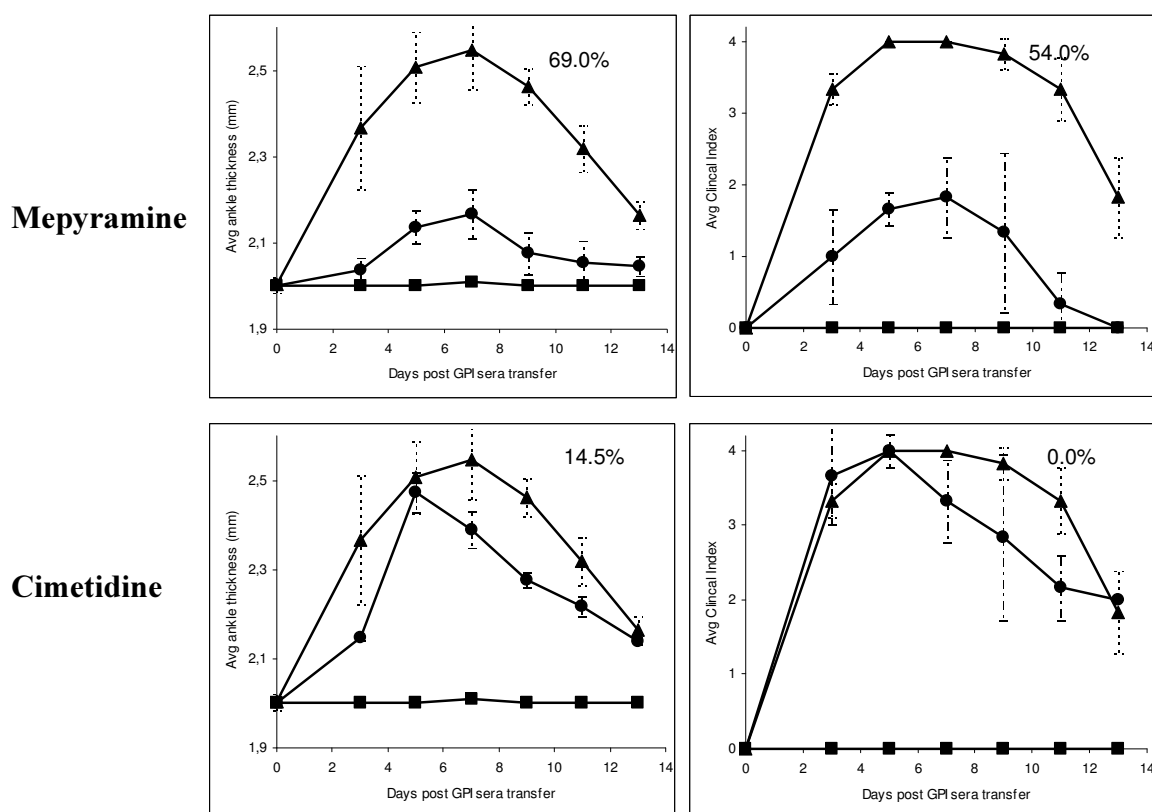
Mast cells generate and store a large number of potent pro-inflammatory mediators such as  $\text{TNF}\alpha$ , vasoactive mediators like histamine, proteases like tryptase and chemokines like IL-8 that play a critical roles in inflammation. During inflammation mast cells degranulate to release these key effector molecules that mediate permeability, inflammation, chemotaxis, and tissue destruction. These cells are also the only cells known to degranulate preformed  $\text{TNF}\alpha$  in granules and also display an ability to rapidly produce large amounts of both  $\text{TNF}\alpha$  and IL-1, cytokines that play a key role in arthritis. Recently Mast cells were shown to play a key role in K/BxN sera transfer model (Lee, Friend et al. 2002).



**Figure 37. Inhibition of mast cell degranulation ameliorates RA.** For Mast cell degranulation inhibition mice were treated with cromolyn or tranilast. Three *i.p* injection cromolyn of each 10 mg (200  $\mu\text{l}$ ) per mice were made on day -1, 0 and 2 with regard to sera transfer. For tranilast three *i.p* injections 250  $\mu\text{g}$  (125  $\mu\text{l}$ ) per mice were made on day -1, 0 and 1 with regard to sera transfer. 100  $\mu\text{l}$  of KBN sera was injected *i.p* 2 hours after the day 0 treatment. The mice were assessed for arthritis development by ankle thickness and clinical index(II) on days 0, 3, 5, 7, 9, 11 and 13. Mice treated with (▲)KBN sera alone, (●) KBN sera and therapy, (■) control sera alone. Data are expressed as mean  $\pm$  SEM; n=3-4 for therapy and K/BxN sera injected mice groups and n=2 for control sera mice group. Percentage reduction in maximum average ankle thickness and maximum clinical index after therapy are shown.

In order to determine the role of mast cell degranulation in K/BxN sera induced RA model BALB/c mice were treated with mast cell stabilizing agents/degranulation inhibitors Cromolyn and Tranilast. The mode of action of Cromolyn is by inhibition of influx of  $Ca^{2+}$  into mast cells. Mice were pretreated with the mast cell degranulation inhibitors and transferred with 100  $\mu$ l of KBN sera on day 0. It was observed that the pre-treatment of the mice with Cromolyn, reduced the maximum average ankle thickness by 63.6% and the maximum clinical index by 56.2% compared to non-treated mice. Similarly the pre-treatment with Tranilast also had an anti-inflammatory effect though not as strong as Cromolyn, with the treated mice showing a 41.8% reduction in maximum average ankle thickness and a moderate 16.75% reduction in maximum clinical index compared to non-treated mice. On the whole the inhibition of mast cell degranulation seemed to ameliorate disease in K/BxN sera transfer model.

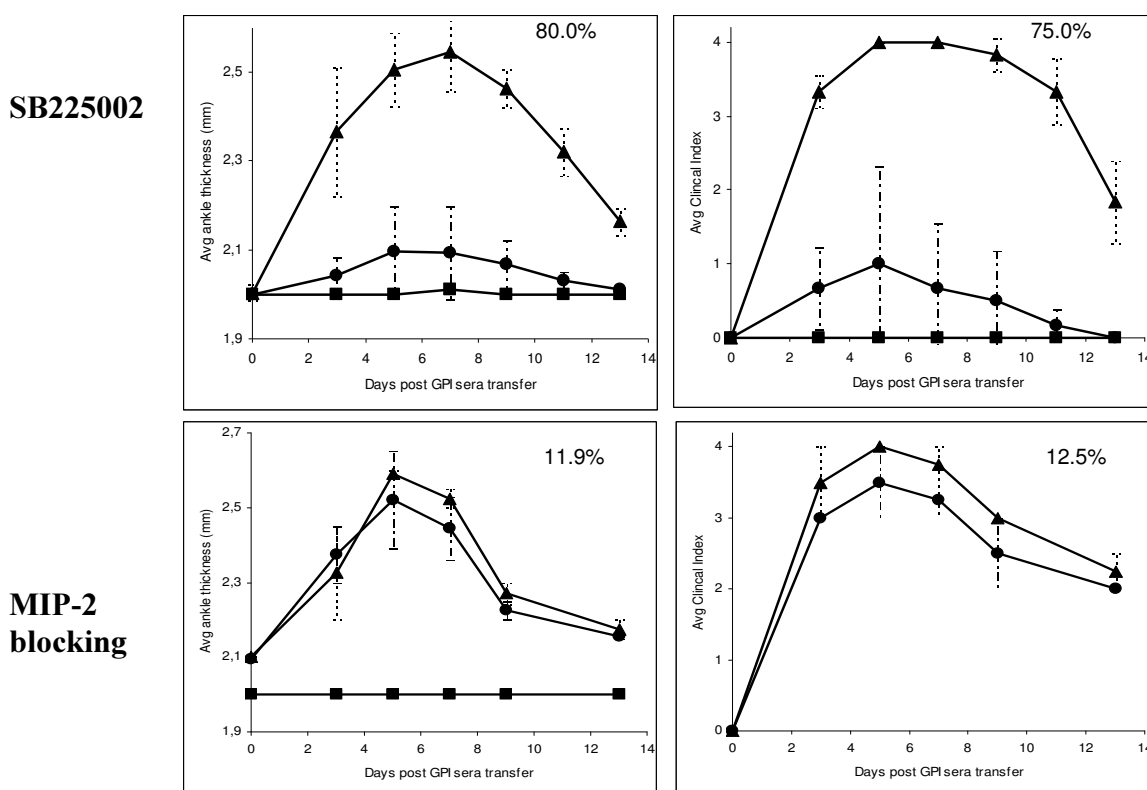
Histamine, is one of the most potent vasoactive factors released from activated mast cells. It acts mainly through three G-protein coupled receptors H1 histamine receptor, H2 histamine receptor and H3 histamine receptor. The H1-histamine receptors are involved mainly in smooth muscle contraction and vascular permeability (Baroody and Naclerio 2000), H2 receptors mediate gastric-acid secretion (Del Valle and Gantz 1997) and H3 receptor regulates histamine synthesis and release from both the central and peripheral nervous system and gastric acid secretion. In order to find a role of histamine receptors in inflammation in the K/BxN sera induced RA murine model, histamine receptor antagonist was administered into BALB/c mice before inducing RA. Mepyramine is a H1 histamine receptor and cimetidine is a H2 histamine receptor antagonist (Kolaczowska 2001). The mice were treated with the antagonist on day -1 and transferred with 100  $\mu$ l of K/BxN sera the next day. We found that the mice treated with H1 histamine receptor antagonist Mepyramine had reduced RA with 69% reduction in the maximum average ankle thickness and a 54% reduction in maximum average clinical index compared to mice not receiving the treatment. The mice similarly treated with H2 receptor antagonist Cimetidine, however did not show such a drastic reduction in ankle thickness and clinical index. The H2 histamine antagonist treatment reduced the maximum ankle thickness by only 14.5% and had no decrease in maximum clinical index compared to non-treated mice. Hence it could be concluded that the effect of histamine in K/BxN induced RA is mainly effected through the H1 histamine receptors while H2 histamine do not have a significant role.



**Figure 38. H1 histamine receptor antagonists ameliorates RA while H2 histamine receptor does not.** Mepyramine, a H1 histamine receptor antagonist or Cimetidine, a H2 histamine receptor antagonist were administered to study the role of histamine and its receptors. Three *i.p* injections of each 450  $\mu$ g in 90  $\mu$ l saline per micewere made with Mepyramine or Cimetidine into each mice on day -1,0 and 2 with regard to sera transfer. 100  $\mu$ l of KBN sera was injected *i.p*, 2 hours after day 0 treatment. The mice were assessed for arthritis development by ankle thickness measurement and clinical index score (II) on days 0, 2, 5, 7, 9, 11 and 13. Mice treated with (▲)KBN sera alone, (●)KBN sera and therapy, (■)control sera alone. Data are expressed as mean  $\pm$  SEM; n=3-4 for therapy and K/BxN sera injected mice groups and n=2 for control sera mice group. Percentage reduction in maximum average ankle thickness clinical index after therapy are shown.

#### 4.4.2. Role of neutrophil recruitment mediators in KBN sera induced RA pathogenesis

Neutrophil influx is predominantly regulated by neutrophil chemokines, such as interleukin (IL)-8 and growth-related oncogene (GRO) in humans, or cytokine-induced neutrophil chemoattractant-1 (CINC-1) and macrophage inflammatory protein-2 (MIP-2, IL-8 in humans)) to the site of acute inflammation in rats/mice. MIP-2 activates neutrophils by binding to two distinct G-protein coupled receptors CXCR1 and CXCR2, while GRO $\alpha$ , GRO $\beta$ , GRO $\gamma$ , and ENA-78 bind to and activate only CXCR2. BALB/c mice were treated with compound SB 225002, a potent, selective non-peptide CXCR2 antagonist to study the role of CXCR2 receptor in neutrophil recruitment.

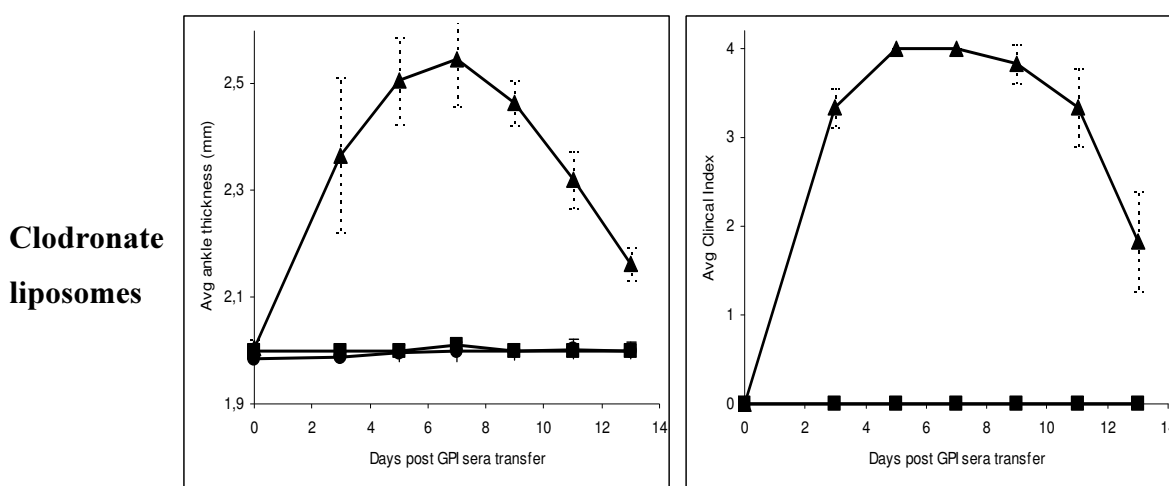


**Figure 39. Role of Neutrophil attracting CXC chemokine receptor. CXCR2 and CXC chemokine MIP-2 in RA.** For CXCR2 antagonism four *i.p* injections each of 80  $\mu\text{g}$  compound SB225002 in 25% DMSO saline per mice were administered on day -1, 0, 2 and 4 with regard to sera transfer. To inhibit MIP-2 two *i.v* injections of rabbit anti-mouse/rat Macrophage inflammatory protein-2 (MIP-2) antibodies at 150  $\mu\text{g}$  (150  $\mu\text{l}$ ) were made in saline on day 0 and 3 with regard to sera transfer. 100  $\mu\text{l}$  of KBN sera was injected *i.p* on day 0 2 hours after antibody administration. The mice were assessed for arthritis development by ankle thickness and clinical index(II) on days 0, 3, 5, 7, 9, 11 and 13. Mice treated with (▲)KBN sera alone, (●)KBN sera and therapy, (■)control sera alone. Data are expressed as mean  $\pm$  SEM; n=3 for therapy and K/BxN sera injected mice groups and n=2 for control sera mice group. Percentage reduction in maximum average ankle thickness clinical index after therapy are shown.

To study the role of MIP-2 in neutrophil recruitment into sites of inflammation injection of BALB/c mice with blocking antibodies to MIP-2 were made. To study the role of CXCR2 receptor, BALB/c mice were treated with IL8R antagonists SB 225002. CXCR2 antagonist injected mice showed a 80% reduction in maximum ankle thickness and 75% reduction in maximum clinical index as compared to untreated mice. This was not the case when mice were treated with blocking antibodies to MIP-2 as the mice only showed a marginal reduction in maximum ankle thickness of 11.9% and maximum clinical index of 12.5%. The lack of reduction in inflammation in MIP-2 blocking may be due to incomplete blocking by our treatment conditions

#### 4.4.3. Role of macrophage and macrophage chemoattractant protein-1 in KBN sera induced RA pathogenesis.

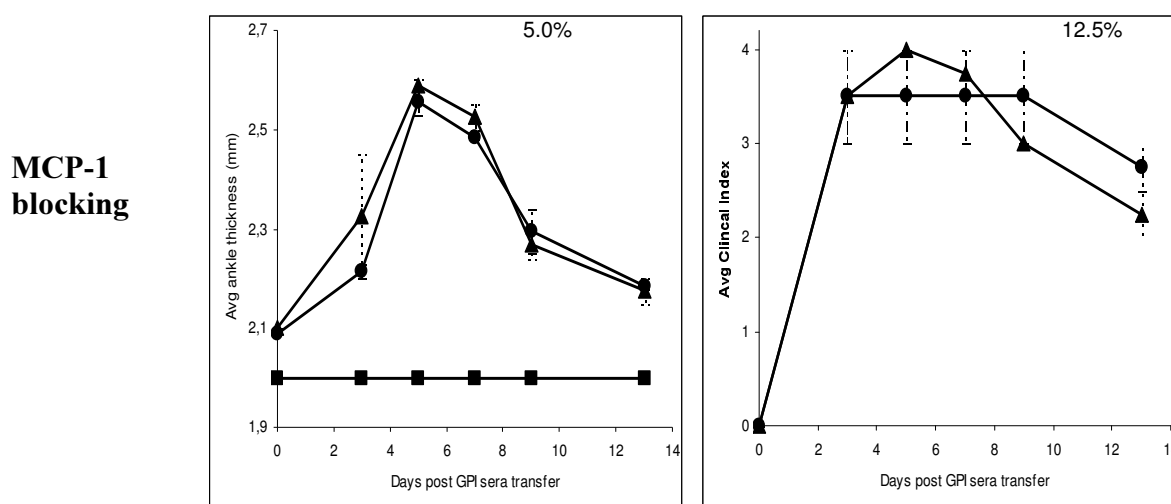
Macrophages have known as inflammatory cytokine factories. These cells are present in high numbers in inflamed tissues and at the cartilage-pannus interface in RA, producing high amounts of inflammatory cytokines  $\text{TNF}\alpha$ ,  $\text{IL1-}\beta$ ,  $\text{IL-8}$ , prostaglandins and tissue degrading proteases-stromelysin, collagenase, gelatinase B and leukocyte elastase. In order to investigate the role of macrophages in K/BxN sera induced RA studies were undertaken in macrophage depleted BALB/c mice.



**Figure 40. Macrophage depleted mice are resistant to RA.** Macrophages were depleted by Clodronate liposomes treatment. Two *i.p.* injections of 200  $\mu\text{l}$  of clodronate liposomes were done on day day-3 and -1 prior to sera transfer was done. 100  $\mu\text{l}$  of KBN sera was injected *i.p.* on day 0, 2 hours after antibody administration. The mice were assessed for arthritis development by ankle thickness and clinical index score (II) on days 0, 3, 5, 7, 9, 11 and 13. Mice treated with (▲)KBN sera alone, (●)KBN sera and therapy, (■)control sera alone. Data are expressed as mean  $\pm$  SEM;  $n=4-5$  for therapy and K/BxN sera injected mice groups and  $n=1$  for control sera mice group.

Depletion of macrophages was achieved by pretreatment of mice with multilamellar liposomes containing dichloromethylene bisphosphonate (clodronate) (Van Rooijen and Sanders 1994; van Rooijen, Sanders et al. 1996). Clodronate is a first generation bisphosphonate which when capsulated within liposomes is able to enter phagocytic cells and initiate apoptosis (Richards, Williams et al. 1999). The mice were depleted of macrophages systemically with two *i.p.* injections of liposome clodronate on days -3 and -1 prior to sera transfer. All the mice pretreated with clodronate liposomes, to deplete the macrophages were completely protected from disease and did not show any signs of RA on K/BxN sera transfer.

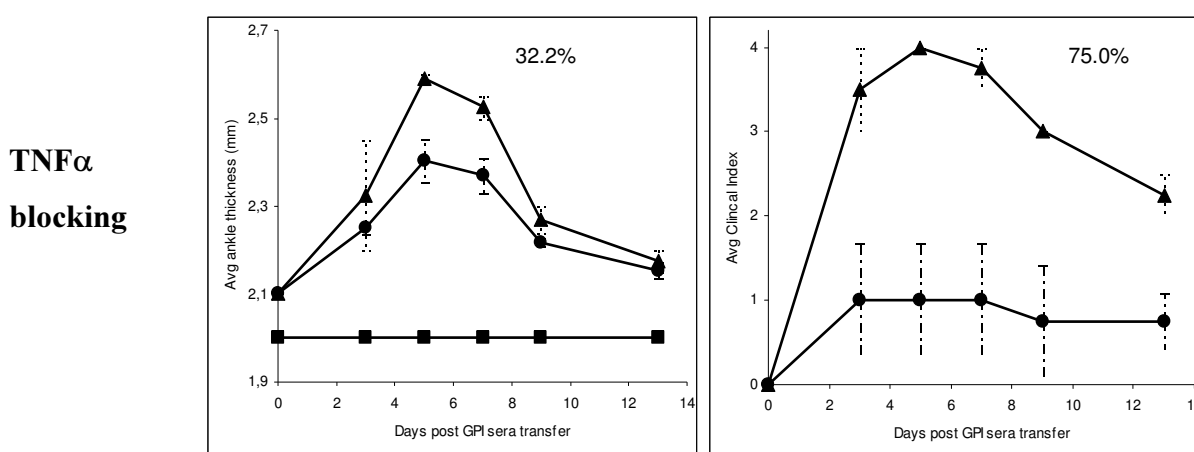
Monocyte chemoattractant protein (MCP)-1 is a chemoattractant cytokine (chemokine) that promotes the migration and activation of monocytes. Recent studies have shown that monocytes are recruited to sites of inflammation through CCR2 receptors by MCP-1 and after differentiation into macrophages and up regulation of CCR1 and CCR5 leads to predominant recruitment by MIP-1 $\alpha$  (Kaufmann, Salentin et al. 2001). In order to see if the treatment with blocking antibodies to MCP-1 would reduce RA in K/BxN sera induced mice, mice were pretreated with MCP-1 antibodies followed by induction of RA with K/BxN sera. It was observed that unlike the complete absence of inflammation seen in case of macrophage depletion only a very moderate 5% reduction in maximum average ankle thickness and a 12.5% reduction in clinical index is seen when compared compared to non treated mice.



**Figure 41. Role of chemokine Macrophage Chemoattractant Protein -1 in RA.** Rabbit anti-mouse/rat Macrophage chemoattractant protein-1 (MCP-1) antibodies were used to block MCP-1 *in vivo*. Two *i.v* injections of 150  $\mu$ g (150  $\mu$ l) were made with the antibodies re-suspended in saline on day 0 and 3 with regard to sera transfer. 100  $\mu$ l of KBN sera was injected *i.p* on day 0, 2 hours after antibody administration. The mice were assessed for arthritis development by ankle thickness and clinical index (II) on days 0, 3, 5, 7, 9 and 13. Mice treated with (▲)KBN sera alone (●)KBN sera and therapy (■)control sera alone. Data are expressed as mean  $\pm$  SEM; n=3-4 for therapy and K/BxN sera injected mice groups and n=1 for control sera mice group. Percentage reduction in maximum average ankle thickness and maximum clinical index after therapy compared to non-treated mice are shown.

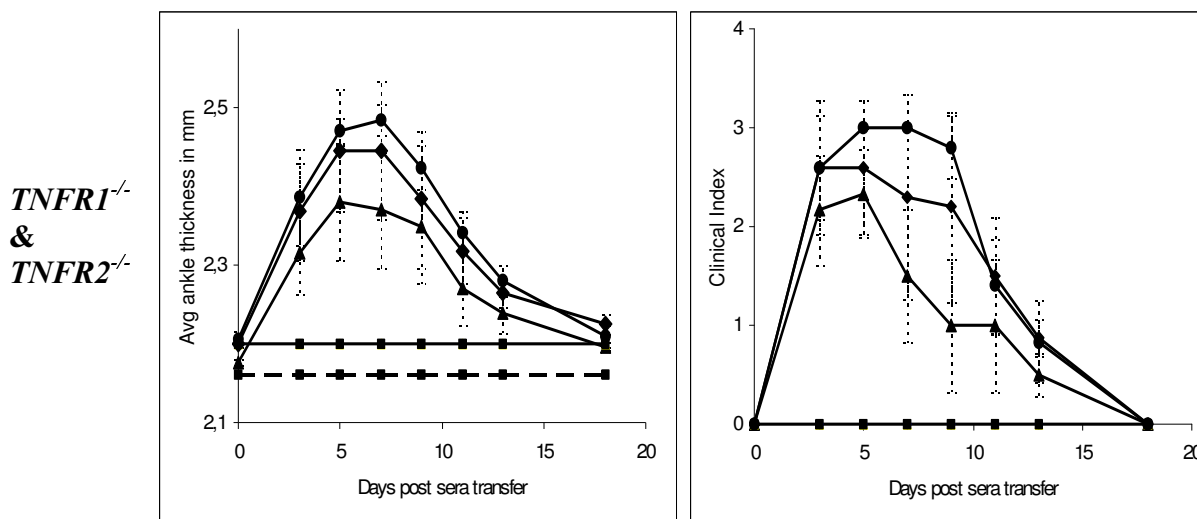
#### 4.5. Role of TNF $\alpha$ and TNF receptors in KBN sera induced RA pathogenesis

TNF is one of the first inflammatory cytokine produced and also usually overproduced in later chronic inflammatory/autoimmune diseases, such as RA. Inhibition of TNF in rheumatoid arthritis (RA) is proving efficacious for a large number of patients (Shanahan and St Clair 2002). Though pharmacological inhibition of TNF in animal models has been successful (Shealy, Wooley et al. 2002). Dual and opposing roles for TNF in chronic autoimmune disease have been recently reported ranging from no or little affect of TNF $\alpha$  therapy (Joosten, Helsen et al. 1999) to exacerbation of inflammation (Campbell, O'Donnell et al. 2001). *TNFR1*<sup>-/-</sup> mice were found to develop CIA with a low incidence and in a milder form (Mori, Iselin et al. 1996). TNFR1 (Pfeffer, Matsuyama et al. 1993) and TNFR2 (Erickson, de Sauvage et al. 1994) gene knockout mice were used to investigate the role of TNFRs in this RA model. In order to study the role of TNF and the TNF receptors in KBN sera induced arthritis TNF $\alpha$  was inhibited *in-vivo* by blocking of TNF $\alpha$  with sheep anti-TNF $\alpha$  antibodies. The sheep anti- mouse TNF $\alpha$  were administered at 500  $\mu$ g/mice/injection on day 0 and day 3 with regard to sera transfer. The mice were injected with relevant sera on day 0 and assessed for arthritis development by ankle thickness, clinical index and joint histology. On pretreatment with TNF $\alpha$  blocking antibodies to K/BxN sera induced RA mice it was found that the mice had a reduction of 32.1% reduction in maximum average ankle thickness and a more striking 75% reduction of maximum clinical index .



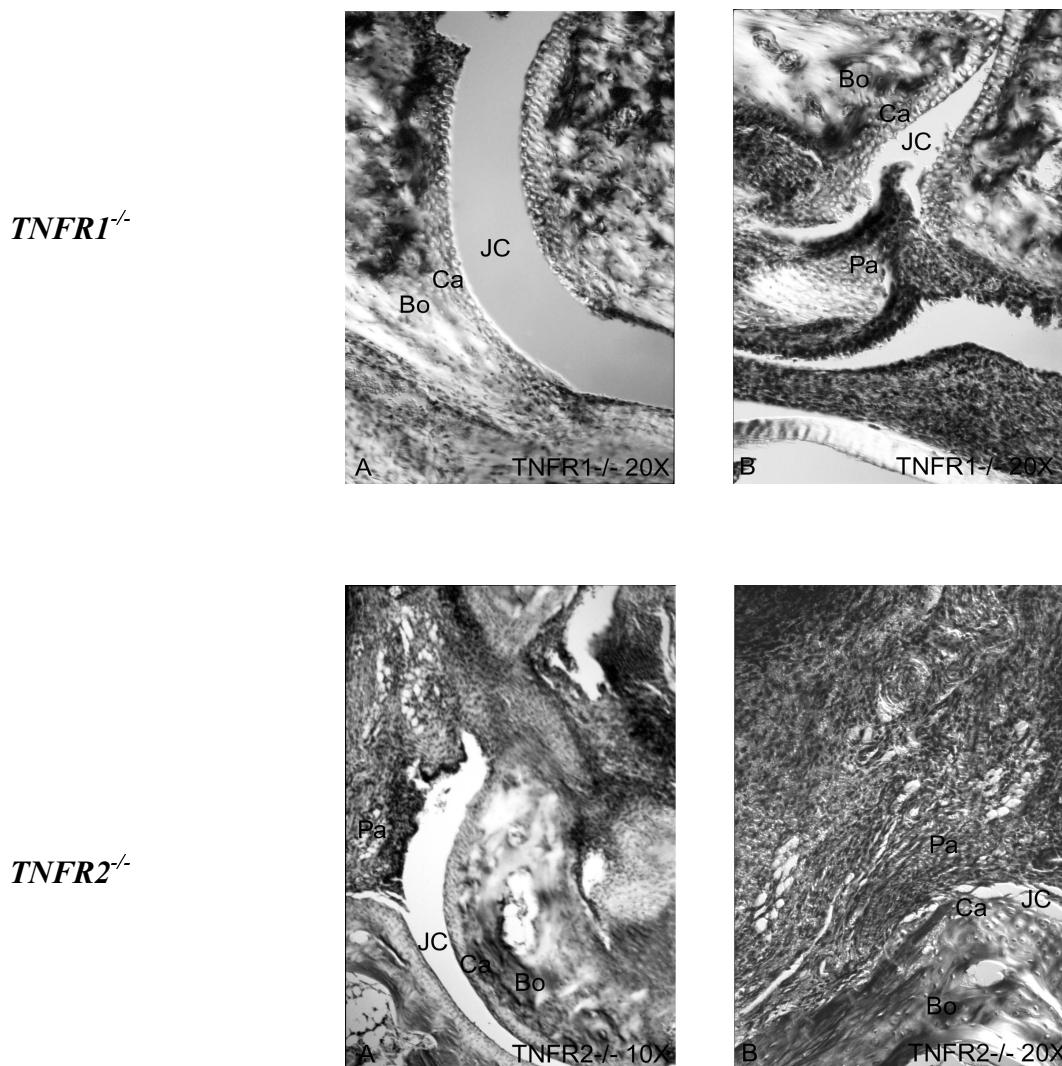
**Figure 42. Neutralizing antibodies to TNF $\alpha$  reduces inflammation in RA.** Two *i.v* injections of 500  $\mu$ g (500  $\mu$ l) each were made with the antibodies re-suspended in saline on day 0 and 3 with regard to sera transfer. 100  $\mu$ l of KBN sera was injected *i.p* on day 0 2 hours after antibody administration. The mice were assessed for arthritis development by ankle thickness and clinical index (II) on day 0, 3 5 7 9 and 13. Mice treated with (▲)KBN sera alone (●)KBN sera and therapy (■)control sera alone. Data are expressed as mean  $\pm$  SEM; n=3-4 for therapy and K/BxN sera injected mice groups and n=1 for control sera mice group. Percentage reduction in maximum average ankle thickness clinical index after therapy are shown.

The  $TNFR1^{-/-}$  and  $TNFR2^{-/-}$  and control C57BL/6 mice were transferred with 100  $\mu$ l of K/BxN sera or control sera and monitored for development of RA by measure of ankle thickness, clinical index and by joint histology.



**Figure 43.  $TNFR1^{-/-}$  and  $TNFR2^{-/-}$  mice are not protected from RA.** The  $TNFR1^{-/-}$  and  $TNFR2^{-/-}$  mice injected intra-peritoneally with 100  $\mu$ l K/BxN or as control with 100  $\mu$ l C57BL/6 serum on days 0. The mice were assessed for average ankle thickness by calliper measurement and a clinical index score on days 0, 3, 5, 7, 9, 11, 13 and 18. K/BxN serum transferred ( $\blacklozenge$ ) $TNFR1^{-/-}$  ( $\bullet$ ) $TNFR2^{-/-}$  ( $\blacktriangle$ )C57BL/6 control sera transferred mice ( $\blacksquare$ ) $TNFR1^{-/-}$  ( $\blacksquare$ ) $TNFR2^{-/-}$  (- $\blacksquare$ -)C57BL/6 are shown for each strain. Data are expressed as mean  $\pm$  SEM; n=4-6 for experimental mice groups and n=2-4 for control mice groups.

Surprisingly we found that the  $TNFR1^{-/-}$  and  $TNFR2^{-/-}$  both developed full blown RA on transfer with K/BxN sera. More surprising was that the  $TNFR1^{-/-}$  and  $TNFR2^{-/-}$  both developed more severe RA with higher ankle thickness and clinical index as compared to C57BL/6 mice transferred with K/BxN sera (figure 43).



**Figure 44. Histology of ankle of *TNFR1*<sup>-/-</sup> and *TNFR2*<sup>-/-</sup> before and after RA induction.** Ankle joints from K/BxN sera and control sera transferred mice at day 11 were processed for H & E staining. Both the *TNFR1*<sup>-/-</sup> and *TNFR2*<sup>-/-</sup> mice showed pannus tissue infiltration into ankle joints leading to cartilage and bone damage. JC-joint cavity; Ca-cartilage; Bo-bone; Pa-pannus tissue.

This also could be confirmed with ankle joint histology (figure 44) as both the *TNFR1* and *TNFR2* deficient mice showed severe inflammation bone and cartilage damage after atransferter K/BxN. Also we found that the *TNFR2*<sup>-/-</sup> had a slightly more severe RA as compared with the *TNFR1*<sup>-/-</sup> mice.