

Review Article

What Have We Learned about the Pathogenesis of Rheumatoid Arthritis from TNF-Targeted Therapy?

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Studies of cytokine regulation in rheumatoid arthritis led to the development of TNF α inhibitors which are now used for a number of indications, including rheumatoid arthritis, inflammatory bowel disease, psoriasis, psoriatic arthritis, and ankylosing spondylitis. The widespread use of biologics in the clinic offers unique opportunities for probing disease pathogenesis and this paper provides an overview of rheumatoid arthritis, with a particular emphasis on the impact of anti-TNF α therapy on pathogenetic mechanisms. An overview is also provided on the most commonly used animal models that mimic RA, including adjuvant-induced arthritis, collagen-induced arthritis, TNF α -transgenic mice, and the K/BxN and SKG models. These models have led to significant discoveries relating to the importance of pro-inflammatory cytokines in the pathogenesis of rheumatoid arthritis, resulting from dysregulation of the normally finely tuned balance of pro- and anti-inflammatory cytokine signalling. In addition, experimental evidence is discussed suggesting how genetic and environmental factors can contribute to disease susceptibility. The role of effector and regulatory T cells is discussed in the light of the relatively disappointing therapeutic effects of T cell modifying agents such as anti-CD4 antibody and cyclosporin. It is concluded that comprehensive analyses of mechanisms of action of biologics and other drugs entering the clinic will be essential to optimise therapy, with the ultimate aim of providing a cure.

1. Overview of Rheumatoid Arthritis

1.1. Introduction. Rheumatoid arthritis (RA) is a chronic, inflammatory disease that can affect multiple tissues and organs, but it principally targets synovial joints. The disease is frequently progressive and results in swelling of joints, pain, and stiffness, with deformity and ankylosis developing in many cases during the later stages. Other features include fatigue, anaemia, and flu-like symptoms. RA has a worldwide prevalence of approximately 1% and is one of the commonest causes of disability in the Western world. The age of onset is typically between 25 and 50, although it can occur at any age. The disease involves inflammation of the capsule surrounding the joints, hyperplasia of synovial cells, oedema, and the development of fibrosis in the synovium. The pathology of the disease process frequently causes destruction of

articular cartilage and ankylosis of the joints. In addition, RA can result in inflammation in the lungs, pericardium, pleura, and sclera as well as subcutaneous nodular lesions. Although the etiology of RA is unknown, it is widely assumed that autoimmune processes play a major role in the initiation and/or perpetuation of the disease.

1.2. Genetic Factors Contributing to Susceptibility. Genetic factors undoubtedly play an important role in determining susceptibility to RA and data from twin studies, in which concordance is approximately 15% [1], suggests that the genetic contribution to disease susceptibility is around 50%. Further analysis has revealed a strong association between susceptibility to RA and MHC class II molecules. Thus, the association between RA susceptibility and specific HLA

DRB1 alleles has been demonstrated in a number of different populations around the world [2]. More recently, genome-wide association studies have identified a number non-HLA RA susceptibility loci which associate with RA, including *AFF3*, *KIF5A*, *PTPN22*, *CTLA4*, *CD40*, 6q23, *TRAF1/C5*, and *STAT4*, and a number of these are involved in lymphocyte signalling [3].

1.3. Potential Environmental Triggers. Genes, however, can only account for about half of the susceptibility to RA and environmental influences are also thought to play a role in the development of the disease. It has been hypothesised that infection is a potential trigger for RA, with mycobacteria, EBV, parvovirus, and a number of retroviruses being suggested as possible candidates. However, despite anecdotal reports, there is a lack of strong epidemiological data in support of an association between RA and infection. In contrast, there is robust evidence of an association between cigarette smoking and the development of RA [4–7]. A similar association has also been described for Crohn's disease.

1.4. The Role of Female Sex Hormones. A puzzling aspect of RA is its tendency to affect women more than men (the female to male ratio is around 3 to 1), which suggests that sex hormones may have an influence on disease. Nevertheless, despite the greater incidence of RA in women, there is a wealth of evidence to suggest a protective effect for female sex hormones. This is based on a number of lines of research, including a number of early studies suggesting that oral contraceptives (consisting of a combination of synthetic derivatives of estrogen and progesterone) are protective in RA [8, 9]. This protective effect appears to be associated more with current contraceptive use than excontraceptive use, as evidenced from a study in which oral contraceptive use was found not to influence disease outcome significantly in the long term, indicating that contraceptives delay, but do not prevent, the development of RA [10].

Another line of evidence implicating the involvement of sex hormones in RA is the fact that pregnancy is usually associated with a reduction in disease activity, whilst the postpartum period is often accompanied by disease flare [12–14]. There is also evidence of increased rate of onset of RA during the postpartum period, especially after the first pregnancy [12–14]. The use of animal models has allowed a more comprehensive analysis of the protective effect of pregnancy. For example, in collagen-induced arthritis (CIA), a well-validated model of RA, disease remission during pregnancy as well as postpartum exacerbation have both been demonstrated [15–17]. Similar findings in rats with adjuvant arthritis and mice with proteoglycan-induced arthritis have been reported [18, 19]. It is significant that pregnancy-induced remission in CIA is associated with a reduction in circulating levels of all type II collagen-specific isotypes of IgG, indicating a profound immunomodulatory effect [20].

In a study designed to explain why there is exacerbation of arthritis after partum, it was found that the prolactin

antagonist, bromocriptine, suppressed the postpartum exacerbation of CIA, as shown by a 50% reduction in disease severity in bromocriptine-treated animals compared to untreated mice. This effect was attributed to suppression of the prolactin release that normally occurs after partum but in a further study by Mattsson et al., it was shown that oestrogen plays an extremely important part in postpartum exacerbation of arthritis [21].

Pregnancy is associated with increased levels of both oestrogen and progesterone and in one study it was shown that estrogen, but not progesterone, was likely to be the critical factor responsible for pregnancy-associated remission of arthritis [22]. This early finding led to the concept that synthetic estrogen receptor agonists would be therapeutically effective in arthritis and this was subsequently confirmed in mice [23–25]. Further analysis indicated that T cell production of IFN γ in lymph node cells was reduced and there was a significant reduction in serum levels of type II collagen-specific IgG2a and an increase in the levels of IgG1 in treated mice. Conversely, blockade of the estrogen receptor, using ICI 182,780, accelerates the onset and increases the severity of CIA [26].

1.5. The Role of T Lymphocytes. The importance of CD4⁺ T cells in the pathogenesis of RA has long been suspected, based on the presence of large numbers of T cells in the joints of RA patients and the well-known association of MHC class II with RA, for which the only known function is to present peptide antigens to CD4⁺ T cells [27]. Additional studies established a robust association in a number of different cohorts between susceptibility to RA and the presence of shared epitopes common to the HLA-DR beta chains of the RA-associated haplotypes [28].

Studies in animal models of arthritis also supported the role of T cells in RA. For example, successful therapeutic intervention was demonstrated using blocking or depleting antibodies, including anti-CD4 [29], anti-TCR [30, 31], anti-IL-2R [32], and anti-MHC class II [33]. However, although these T cell-targeted therapies were successful in animal models (usually when administered prophylactically rather than therapeutically), clinical trials of depleting anti-CD4 mAb therapy in RA were in general disappointing, despite achieving a high rate of CD4⁺ blood T cell depletion [34, 35]. Some nondepleting anti-CD4 mAbs were shown to provide transient beneficial effects [35] but the relatively low level of efficacy combined with the occurrence of side effects (e.g. vasculitis) has hampered the further clinical development of this therapeutic strategy.

Other therapeutic approaches that have been tested targeting T cells in RA have included cyclosporin, FK-506, rapamycin [36], and CAMPATH-1H [37], all of which act in a relatively indiscriminate fashion. However, it is now becoming clear that healthy individuals possess a major CD4⁺ T cell subset, known as regulatory T cells, that protect against inflammation and autoimmunity. Hence one of the possible reasons for the failure of these therapies is that they target both regulatory as well as effector T cell subsets. More recently, CTLA4-Ig, which blocks T cell costimulation, has

proven to be efficacious in RA, confirming the importance of T cells in driving the disease [38].

1.6. Does Aberrant MHC Class II Expression Play a Role in the Pathogenesis of RA? As discussed above, HLA class II molecule expression is strongly up-regulated in RA, as well as a number of other human autoimmune diseases. In some cases, such as Graves' autoimmune hyperthyroidism, the increased MHC class II expression is seen not only on professional antigen presenting cells (APCs), such as dendritic cells, but also on cells that do not usually express MHC class II molecules, for example, thyroid epithelial cells [39, 40]. In RA it was shown that endothelial cells and fibroblasts in the joint expressed increased MHC class II molecules and this was regarded as evidence of increased APC activity [27, 41]. Since it was known that cytokines, such as IFN γ , were the principal inducers of up-regulated MHC class II expression, it was hypothesized that over-expression of cytokines coupled with increased APC function was an important factor in the pathogenesis of autoimmune disease [39]. Furthermore, evidence accrued suggesting that increased APC function was indeed important in Grave's disease, an organ-specific autoimmune disease that targets the thyroid, leading to hyperthyroidism [42, 43].

2. Animal Models: What Can They Teach Us?

2.1. Introduction. Animal models of RA have been used widely for preclinical testing of novel therapies, analyses mechanisms of drug action, the identification of both pro- and anti-inflammatory mediators, and the analysis of genetic susceptibility factors. A brief overview of the most widely studied models and what they can teach us about RA is provided below.

2.2. Adjuvant Arthritis. Adjuvant arthritis is induced in rats by a single injection of complete Freund's adjuvant (CFA) [44]. Onset of arthritis is around 10–45 days after injection and generally subsides after approximately about 30 days. The histopathological features of the disease include oedema, infiltration into the joint of mononuclear and polymorphonuclear cells, and periostitis with erosion of cartilage and bone.

A number of studies have suggested an association between immunity to 65-kDa heat shock proteins and the induction of adjuvant arthritis [45] but no single mycobacterial immunogen has been shown to be responsible for the disease [46]. One suggestion is that the induction of adjuvant arthritis is due to a mycobacterial cell wall component, muramyl dipeptide, which stimulates the innate immune system but does not evoke a specific immune response [47]. Furthermore, evidence accrued suggests that a number of adjuvants completely lacking immunogenicity are capable of causing arthritis in genetically susceptible strains of rats, including avridine [48], incomplete Freund's adjuvant and pristane [46]. In general, adjuvant arthritis is not observed in mice although a form of arthritis has been reported in mice after injection with pristane [49]. Any doubts regarding the

immunological nature of adjuvant arthritis were dispelled by the findings that (i) anti-T cell treatments prevent arthritis [46, 50], (ii) susceptibility is associated with MHC class II genes [51, 52] and (iii) the disease can be adoptively transferred by T cells [53, 54].

Although the basic mechanism underlying the pathogenesis of adjuvant arthritis is unknown, a likely explanation is that injection of adjuvants causes an increase in the activity of APC [56], leading to presentation to self-reactive T cells of a hitherto unrecognised or "sequestered" autoantigen. This is supported by the fact that the onset of joint adjuvant arthritis coincides with the peak of the T cell response. From these models of arthritis we can conclude that autoimmunity can arise from non-specific activation of the innate immune system and it is an open question whether human RA could also be triggered by environmental stimuli. It is of interest to note that in one study, arthritis was observed in susceptible DA rats following percutaneous exposure of adjuvant oils [57], and even a mineral oil-containing cosmetic product [58]. However, perhaps the most likely environmental stimulus with the potential to act as an adjuvant is infection, although a link between infection and RA has yet to be established.

2.3. Collagen-Induced Arthritis (CIA). The CIA model has been widely studied as a model for RA, largely based on its pathological similarities to RA [59, 60]. In addition, susceptibility to both diseases is strongly linked to MHC class II genes. Thus, susceptibility to CIA is mainly restricted to mouse strains bearing MHC types I-A^d and I-A^r whereas susceptibility to human RA is associated with certain subtypes of DR4 and DR1 [59]. The majority of knockout strains of mice are on a C57BL/6 background (H-2^b), which is generally regarded to be resistant to CIA. Hence, one way to study the impact of a gene deletion is to backcross the gene knockout strain onto the DBA/1 background but this is costly and time consuming. However, it has recently been shown that it is possible to induce arthritis in C57BL/6 mice [61–63], which greatly improves the opportunities to use the model for studying disease pathogenesis.

CIA is induced by immunisation of susceptible strains of rats and mice with type II collagen in CFA. The pathological changes include synovitis, pannus formation, erosion of bone and cartilage, and fibrosis (Figure 1). CIA was previously thought to be a Th1-mediated disease, on the basis of the high levels of IFN γ expression during disease induction [64]. However, more recent research points to a more important role for Th17 cells than Th1 cells in disease induction and/or progression [65] and evidence is also emerging of a major role for Th17 cells in human RA.

In CIA, joint inflammation generally occurs after the peak of the T cell response, that is, when the T cell response is diminished, similar to RA. Another important similarity between human RA and murine CIA is that autoantibodies are known to play an important role in the pathogenesis of both CIA and RA [59]. However, whilst the specificity of the autoantibody response in CIA is directed towards type II collagen, there is a lack of convincing data pointing to a

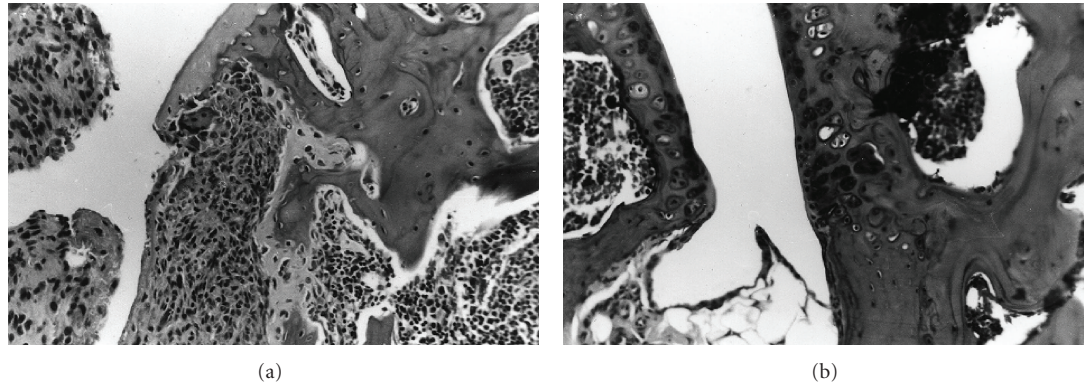


FIGURE 1: Erosive changes in CIA. Top: proximal interphalangeal joint of a mouse with CIA showing marginal bone erosion and loss of chondrocytes from the cartilage. Bottom: normal joint. Haematoxylin and eosin.

role for type II collagen autoimmunity in the majority of RA patients, where only 10–15% of RA patients have antibodies to type II collagen [66].

Another important feature of CIA that resembles RA is the expression of pro-inflammatory cytokines, in particular $\text{TNF}\alpha$ and $\text{IL-1}\beta$ in the joints of mice with CIA [67], and the fact that antagonism of these molecules reduces the severity of arthritis [68–75].

Despite these similarities, there are also important differences between murine CIA and human RA. For example, CIA induced by immunisation with heterologous (foreign) type II collagen is a relatively acute disease in which arachidonic acid metabolites, such as prostaglandin E_2 , play an important pathological role in the disease. This was illustrated by the results of a study of CIA in cytosolic phospholipase $\text{A}2\alpha$ ($\text{cPLA}2\alpha$) knockout mice that had been backcrossed onto a DBA/1 background. $\text{cPLA}2\alpha$ releases arachidonic acid from cell membranes (the first step in the production of prostaglandins and leukotrienes). The progression of CIA was markedly inhibited in $\text{cPLA}2\alpha$ -deficient mice compared with wild-type DBA/1 mice, in spite of the fact that serum levels of type II collagen specific antibodies were similar in the two groups [76]. On the other hand, immunisation of DBA/1 mice with homologous (mouse) rather than heterologous (foreign) type II collagen results in a more chronic form of arthritis [77–79], which more closely resembles human RA in terms of its clinical course and its relative lack of response to non-steroidal anti-inflammatory drugs (NSAIDs), such as indomethacin [79]. Similarly, it was recently shown that immunisation of C57BL/6 mice with chicken type II collagen results in a relatively chronic form of arthritis with a more sustained T cell response than the conventional CIA model [80]. In this study C57BL/6 were immunised with type II collagen using different protocols and arthritis incidence, severity, and response to commonly used antiarthritic drugs were assessed and compared with DBA/1 mice. C57BL/6 mice were found to be susceptible to arthritis induced by immunization with chicken type II collagen (but not bovine collagen) and developed strong and sustained T cell responses to type II collagen. Arthritis was milder in C57BL/6 mice than in

DBA/1 mice but more closely resembled rheumatoid arthritis in its response to therapeutic intervention [80].

2.4. *hTNF-Transgenic Mice.* The group of Kollias et al. generated a strain of mice over-expressing a human $\text{TNF}\alpha$ transgene (including its endogenous promoter region), that had been disregulated by the replacement of the 3' AU rich region (which confers mRNA instability) with the 3' untranslated region of the human β -globin gene (Figure 2). Despite not being targeted to any particular cell type, $\text{hTNF}\alpha$ transgenic mice were shown to develop a spontaneous form of arthritis [11]. The disease could be prevented by continuous administration of anti-human $\text{TNF}\alpha$ mAb, confirming the role of the transgenically expressed $\text{TNF}\alpha$ in the induction of arthritis. Analysis of the joints of $\text{hTNF}\alpha$ transgenic mice revealed histopathological similarities to human RA and demonstrated that the disease was highly erosive in nature, with sub-chondral bone, rather than cartilage, being mostly affected (Figure 3).

$\text{TNF}\alpha$ expression in $\text{hTNF}\alpha$ transgenic mice was not confined to the joint and was found to be overexpressed in various tissues, including lung, spleen, and the joint. The reason why the joint should be affected whilst other tissues are spared is not known and, in fact, a second strain of $\text{TNF}\alpha$ transgenic mice was generated in which the $\text{TNF}\alpha$ transgene lacked an AU rich region. These mice were found to develop, not only arthritis, but also inflammatory bowel disease [81]. It is interesting to note that $\text{TNF}\alpha$ overproducing mice can be backcrossed to $\text{RAG}^{-/-}$ mice without altering the arthritis phenotype, indicating that the adaptive immune system plays no role in the development of arthritis in this model. In contrast, the inflammatory bowel disease was downregulated in $\text{TNF}\alpha$ -transgenic $\text{RAG}^{-/-}$ mice, indicating the involvement of lymphocytes in this model [81].

A significant finding was that treatment of $\text{hTNF}\alpha$ transgenic mice with a blocking anti-IL-1R antibody prevented the development of spontaneous arthritis [82], demonstrating that IL-1 is an important downstream pathological mediator in this model. This is consistent with findings in human RA synovial cell cultures in which $\text{TNF}\alpha$ inhibition

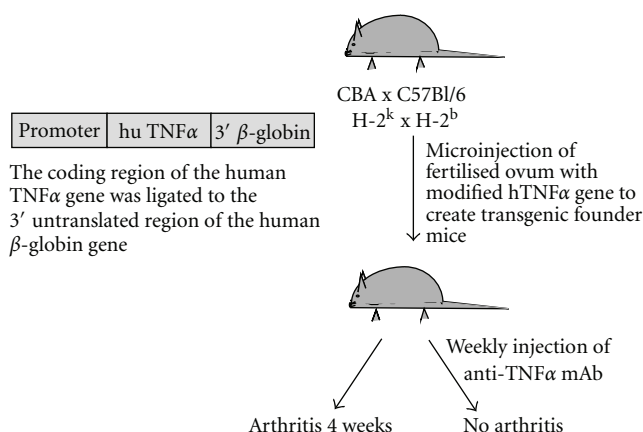


FIGURE 2: Generation of hTNFα transgenic mice. The replacement of the 3' AU rich region of the TNFα gene by the 3' region of the β-globin gene results in overexpression of TNFα protein [11].

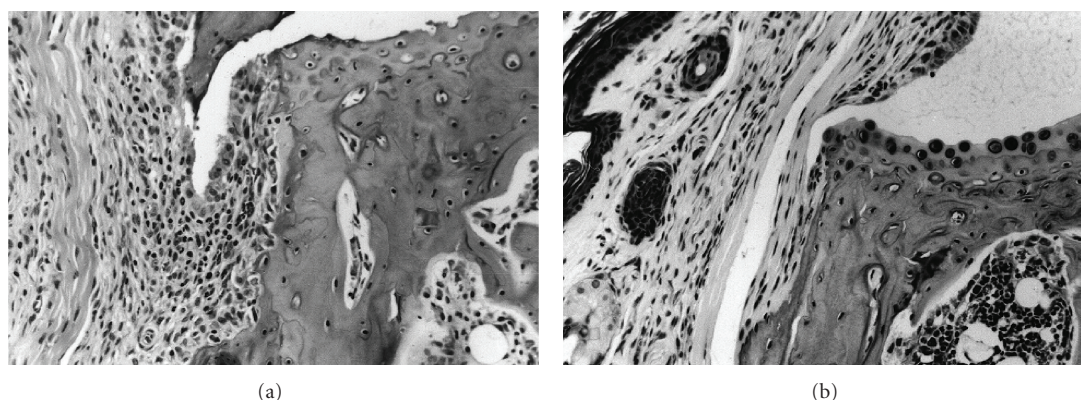


FIGURE 3: Joint damage in hTNFα-transgenic mice. Top: erosive changes in the cartilage-bone-pannus region of a proximal interphalangeal joint from a hTNFα-transgenic mouse with arthritis. Note the focal erosion of subchondral bone. Bottom: normal joint from a non-transgenic littermate. Haematoxylin and eosin.

was found to block IL-1 production [83], indicating the dependence of IL-1 production on TNFα.

2.5. hIL-1α-Transgenic Mice. Another transgenic model that confirms the arthritogenic potential of IL-1 is the IL-1α-transgenic mouse which spontaneously develops severe arthritis at around one month of age [84]. Synovitis was first seen at two weeks of age and synovial lining layer hyperplasia and pannus formation were seen at two months. Unlike TNFα-transgenic mice, severe degradation of cartilage was observed in IL-1α-transgenic mice, thereby confirming previous findings, published over a quarter of a century ago, regarding the important role of IL-1 in cartilage breakdown [85–87].

The cellular infiltrate in the joints of IL-1α-transgenic mice is dominated by neutrophils with few T or B lymphocytes. This emphasises the fact that abnormal activation of innate immunity is sufficient for initiation of arthritis and that acquired immunity may not be crucial for RA.

It is evident from both the hTNFα- and hIL-1α transgenic strains of mice that the over-expression of pro-inflammatory cytokines can drive arthritis despite the fact

that the over-expression is not confined to the joint, suggesting that the joints are particularly sensitive to the effects of pro-inflammatory stimuli.

2.6. The K/BxN Model of Arthritis. Kouskoff et al. described an intriguing model of arthritis in the offspring of KRN TCR-transgenic mice crossed with NOD mice [88]. KRN TCR transgenic mice expresses a TCR specific for an epitope of bovine pancreas ribonuclease presented in the context of I-A^k [89]. However, it was found by chance that when KRN mice are crossed with NOD mice (I-A^{g7}), the resulting (K/BxN) offspring develops arthritis (mainly affecting distal joints) spontaneously at approximately 4–5 weeks of age. Subsequent studies showed that arthritis in K/BxN mice was dependent on I-A^{g7} MHC class II molecules and could be prevented by anti-CD4 mAb treatment [88, 90, 91]. Although this shows that the disease is dependent on CD4⁺ T cells, it was also found that B lymphocytes were required for arthritis development [88, 90]. In addition, arthritis could be transferred by injecting naive mice with serum IgG from arthritic mice in a complement-dependent and FcγR-dependent manner, which demonstrates the important role

played by autoantibodies in this model [90, 92, 93]. It was also shown that the molecular target of the autoantibodies in K/BxN mice was glucose-6-phosphate isomerase (GPI), presented in the context of I-A^{g7} MHC class II molecules [94].

GPI is a ubiquitous cytoplasmic enzyme therefore an important question arising from this model is how can a joint-specific autoimmune disease arise from autoreactivity to an antigen that is not expressed specifically in the joint? In fact the answer to this question was provided by the results of an immunohistological study of the joints of K/BxN mice, which showed accumulation of extracellular GPI on the lining of the articular cavity, particularly on the cartilage surface [95]. The accumulation of GPI on cartilage was more pronounced in arthritic K/BxN mice and GPI staining was found to colocalize with IgG and the C3 component of complement. It was hypothesised on the basis of these findings that complexes of GPI and anti-GPI are responsible for the development of arthritis by initiating a complement-mediated inflammatory cascade in the joint [95]. A lesson to be learnt from the K/BxN model is that arthritis may arise as a result of an immune response to an antigen that is not found specifically in the joint and therefore the common assumption that RA results from autoimmunity to a joint-specific antigen may not be valid.

However, the KRN model differs from human RA in one important respect: its lack of dependence on TNF α [96]. Another point that is worth emphasising is that GPI is unlikely to be the critical autoantigen in RA as antibodies to GPI are no more common in RA than in other diseases [97, 98], despite one report identifying a potential association between anti-GPI antibodies and RA [99].

2.7. SKG Mice. Sakaguchi et al. it showed that a spontaneous (loss of function) point mutation of the gene encoding an SH2 domain of ZAP-70 resulted in the development of chronic autoimmune arthritis in mice [100]. The result of this is that T cells in SKG mice respond weakly to antigenic stimulation and it is proposed that suboptimal signal transduction via the TCR as a result of aberrant ZAP-70 leads to a change in the threshold of T cells to thymic selection, resulting in a failure to delete self-reactive T cells. Again, this model challenges another commonly held belief that autoimmune diseases arise as a result of hyper-responsive T cells. It will be of interest to establish whether a similar phenomenon could contribute to the development of human autoimmune disease.

Another interesting facet of the SKG model of arthritis is the requirement for stimulation through the innate immune system, which can be provided by housing the mice in a microbially rich environment or by administration of fungal β -glucans, such as zymosan [101]. This elegantly demonstrates how genetic factors can interact with environmental stimuli to generate autoimmune disease and provides a valuable model for further study.

2.8. Genetics of Arthritis Susceptibility: The Use of Animal Models. As discussed above, susceptibility to human RA is

strongly influenced by MHC class II region genes although non-MHC genes may also contribute to disease susceptibility as well as disease severity. The identification of genes contributing to susceptibility/severity would provide important clues regarding the aetiopathogenesis of human RA. However, environmental variability and genetic heterogeneity make the identification of specific genetic loci determining susceptibility to RA extremely difficult in humans. However, the impact of environmental differences and genetic heterogeneity can be minimised or even eliminated through the use of animal models using inbred strains. In one study, quantitative trait loci (QTL) controlling susceptibility to arthritis were mapped in the offspring of resistant versus susceptible inbred strains of rat. Not unexpectedly, a major susceptibility QTL was identified within the MHC region and the authors subsequently delved deeper into the question by comparing arthritis severity in rats bearing arthritis-susceptible MHC genotypes [102]. Four QTLs influencing the severity of arthritis were identified outside the MHC class II region (on chromosomes 1, 4, 7, and 10) [102].

In another study involving pristane-induced arthritis, 15 QTLs were identified that contributed to susceptibility to disease [103–105]. By positional cloning of one of these loci, a naturally occurring polymorphism of *Ncf1* contributing to arthritis severity was identified [106]. *Ncf1* encodes neutrophil cytosolic factor 1, a component of the NADPH oxidase complex that is found in phagocytic cells, including macrophages, therefore is of particular interest. Subsequently it was shown that the disease-related allele of *Ncf1* was associated with a sub-optimal degree of oxidative burst which had the unexpected consequence of promoting the activation of arthritogenic CD4⁺ T cells. It was also found that administration of phytol (an activator of the NADPH oxidase complex) resulted in a reduction in the severity of arthritis when given during the induction phase of the disease [106]. These findings point to an important role for *Ncf1* in pristane-induced arthritis and, by extension, may also be true for other forms of arthritis, including RA.

3. Proinflammatory Cytokines in RA

3.1. The Role of Pro- and Anti-Inflammatory Cytokines. Although there have been advances in molecular medicine over the past few years, progress towards identifying the cause of RA has been painfully slow. In contrast, there has been major progress in unravelling the pathological processes that occur during the course of the disease. An example of this would be the identification of the multitude of cytokines, chemokines, and growth factors involved in the maintenance of the chronic inflammation in RA (Table 1). In particular, two cytokines, TNF α and IL-1, have been demonstrated to be important inducers of both inflammation and erosion of cartilage and bone. In a pivotal set of pioneering experiments, carried out in the late eighties and early nineties by Professor Fionula Brennan (now sadly deceased), it was shown that blockade of TNF α in cultures of dissociated synovial cells from RA patients caused the downregulation of the expression of IL-1, IL-6, IL-8, and GM-CSF [83, 107,

TABLE 1

Cytokine	Expression	
	mRNA	Protein
Proinflammatory		
IL-1 α , β	+	+
TNF	+	+
Lymphotoxin	+	\pm
IL-6	+	+
GM-CSF	+	+
M-CSF	+	+
LIF	+	+
Oncostatin M	+	+
IL-12	+	+
IL-15	+	+
IFN α , β	+	+
IFN γ	+	\pm
IL-17	+	+
IL-18	+	+
Immunoregulatory		
IL-2	+	\pm
IL-4	\pm	–
IL-10	+	+
IL-11	+	+
IL-13	+	+
TGF β	+	+
Chemokines		
IL-8	+	+
Gro α	+	+
MIP-1	+	+
MCP-1	+	+
ENA-78	+	+
RANTES	+	+
Growth factors		
FGF	+	+
PDGF	+	+
VEGF	+	+

Cytokines expressed in synovial tissue from patients with RA. Adapted from [55].

108], which is indicative of a cytokine cascade whereby TNF α is responsible for driving the production of multiple cytokine mediators of inflammation.

However, although it is tempting to focus exclusively on the expression of pro-inflammatory cytokines the possibility of a deficiency in the appropriate level of expression of anti-inflammatory cytokines should also be seriously considered. However, this appears not to be the case as a variety of anti-inflammatory cytokines has now been demonstrated to be upregulated in the joints of RA patients, including IL-10 [109], IL-11 [110], soluble TNF receptor [111, 112], IL-1 receptor antagonist [113], and TGF β [114]. Similarly, in the CIA model IL-10, IL-1Ra, TGF β 1, TGF β 2, and TGF β 3 were all found to be abundantly expressed in the joints of mice with [115]. These anti-inflammatory cytokines are detected

in human synovial cell culture supernatants at biologically significant concentrations and are fully functional, as indicated by the fact that neutralisation of IL-10 or IL-11 in synovial cultures resulted in an increase in the production of IL-1 and TNF α [109, 110]. In the light of these findings it was hypothesised that chronic inflammation in RA is due to the fact that the cytokine equilibrium is dysregulated, such that there is a dominance of pro-inflammatory cytokines over the anti-inflammatory cytokines.

3.2. What Is the Effect of TNF α Blockade? Early in vivo evidence for a role for TNF α in driving joint inflammation was provided by the finding of spontaneous arthritis in hTNF-transgenic mice [11]. It was then shown that blockade of TNF α in CIA reduced the clinical severity of arthritis and reduced the degree of joint erosion [69, 74]. Subsequently, the importance of TNF α in the pathogenesis of human RA was confirmed in clinical trials in which intravenous administration of chimeric anti-TNF α mAb (infliximab, Remicade) reduced disease activity and radiographic progression of disease [116–118]. Similar results were later reported for soluble TNF receptor-Fc fusion protein (etanercept, Enbrel) [119–121]. These trials were important in confirming the importance of TNF α in RA and also provided an excellent opportunity to address questions regarding disease pathogenicity in relation to TNF α over-expression.

3.3. The Role of Angiogenesis. Angiogenic factors are thought to play important roles in a number of pathological conditions, including tumour growth and metastasis, diabetic retinopathy, age-related macular degeneration, atherosclerosis and inflammation. Hence the obvious question has been asked whether angiogenesis plays a role in RA. Indeed, early changes in the synovium are characterized by synovial angiogenesis as well as inflammatory cell infiltration and synoviocyte hyperplasia and some recent studies suggest that angiogenesis precedes many of these other pathological features of the disease [122]. Clearer evidence of a role for angiogenesis in the pathogenesis of RA is provided by the observation that a high level of angiogenesis is invariably observed in synovial tissue from RA patients with active disease [123] and by the fact that synovial hyperplasia, infiltration of mononuclear cells, and the degree of severity of joint tenderness correlate with the number of synovial blood vessels [124].

Consistent with these findings, a number of proangiogenic cytokines and growth factors have been identified in the joints of RA patients [125] and serum levels of vascular endothelial growth factor (VEGF, which is generally regarded as a “master regulator” of angiogenesis) are elevated in RA compared to healthy controls or patients with osteoarthritis [126]. It was also shown that serum VEGF levels when patients first presented at the clinic correlated with the degree of joint deterioration seen in radiographs during the first year, indicating that serum VEGF levels are valid predictors of disease progression [126].

A significant finding to come out of the clinical trials of anti-TNF α biologic therapy of RA was the reduction

of serum VEGF levels after therapy. In addition, when anti-TNF α mAb was administered in combination with methotrexate the period of reduced VEGF levels compared to anti-TNF α alone was significantly extended [127]. These findings suggest that at least one of the mechanisms of TNF α blockade is the reduced level of VEGF expression, resulting in a reduction in angiogenesis and consequent amelioration of disease. This is consistent with the observation that blockade of VEGF in established CIA reduces joint inflammation [128–130].

3.4. Leukocyte Infiltration. There is abundant evidence indicating that an important mechanism of action of anti-TNF α is the interruption of inflammatory processes via a reduction in the number of cells infiltrating the joint. For example, early data supporting this hypothesis were provided by a study in which radiolabelled granulocytes were used to monitor cellular infiltration in RA patients treated with anti-TNF α antibody (infliximab) [131]. A group of 10 RA patients with longstanding RA were given a single intravenous infusion (10 mg/kg) of anti-TNF α and the accumulation of autologous granulocytes (separated *in vitro*, labelled with ^{111}In and then reinjected) in the joint was measured by gamma-camera imaging before and after therapy. In addition, synovial biopsy samples were also assessed at the same sequential time points with the aim of measuring numbers of infiltrating CD3 $^{+}$ T cells, CD22 $^{+}$ B cells, and CD68 $^{+}$ macrophages by immunohistochemistry. Synovial tissue was also studied in parallel for the expression of the chemokines: IL-8, MCP-1, MIP-1 α , MIP-1 β , Gro α , and RANTES. Serum levels of IL-8 and MCP-1 were also measured.

The numbers of infiltrating inflammatory cells were shown to be reduced by about 50% in the joints after a single injection of infliximab as illustrated by the fact that there was a significant reduction in the infiltration of ^{111}In -labelled granulocytes into arthritic joints and a significant reduction in infiltrating T cells, B cells, and macrophages. These reductions were paralleled by a significantly reduced level of expression of IL-8 and MCP-1, confirming the role of TNF α in driving chemokine expression [131].

3.5. Altered T Cell Signalling: A Cause or Consequence of RA? As discussed above, autoimmunity arises in the SKG mouse as a result of blunted TCR signalling and although most efforts to explain RA have focussed on the presence of pro-inflammatory pathways, it is also possible that deficiencies in inhibitory or anti-inflammatory pathways contribute to the pathogenesis of the disease. One intriguing possibility that has been explored by Cope is that prolonged exposure of T cells to TNF α results in the induction of hyporesponsiveness to TCR ligation and the dampening of TCR signal transduction pathways. This could, in turn, lead to a failure in T cell-driven immunoregulatory cytokine circuits (e.g. regulatory T cells), whilst at the same time reducing activation-induced cell death and promoting effector responses [132]. To test this hypothesis, tetanus toxoid-specific T cell clones were chronically exposed to

TNF α for up to 16 days before rechallenge with tetanus toxoid antigen. Proliferative responses were found to be reduced by chronic TNF α stimulation in a dose- and time-dependent fashion although responses to IL-2 and PHA were maintained [133]. Chronic exposure to TNF α also reduced the production of IL-2, IL-10, IFN γ , TNF α , and lymphotoxin following stimulation with anti-CD3 antibody and reduced the level of expression of IL-2R α chain. Conversely, blockade of TNF α in T cell cultures enhanced proliferation and cytokine production and promoted the expression of IL-2R α following mitogenic stimulation with anti-CD3 mAb.

These findings point to a role for TNF α regulating T cell activity in RA. To validate this hypothesis, T cell responses were analysed in RA patients undergoing therapy with anti-TNF mAb. TNF α blockade resulted in restoration of the T cell proliferative responses to mitogens as well as to recall antigens in all patients tested, confirming that chronic over-expression of TNF α impacts upon cell-mediated immune responses [133].

3.6. The Relationship between TNF and Regulatory T Cells. There is a considerable degree of controversy surrounding the relationship between TNF and regulatory T cell responses. On the one hand, numerous reports have documented the inhibitory effect of inflammation, and more specifically TNF, on numbers and/or function of regulatory T cells. For example, in a seminal study by Ehrenstein et al. it was shown that regulatory T cells from RA patients were rendered incapable of modulating pro-inflammatory cytokine production by effector T cells stimulated with anti-CD3 [134]. Importantly, treatment with anti-TNF α mAb led to an increase in numbers of circulating regulatory T cells and restored their suppressive activity. Subsequently it was shown that TNF blockade in RA led to the emergence of a FoxP3 $^{+}$ regulatory T population that mediates suppression via TGF β and IL-10 [135]. In a separate study, increased spontaneous apoptosis of regulatory T cells was demonstrated in patients with active RA and this was reduced by treatment with TNF blocking mAb [136]. Consistent with these findings, it was reported that regulatory T cell suppressor function was compromised in RA by myeloid cell-derived inflammatory mediators [137]. Further studies have confirmed that suppressor cell function of regulatory T cells is defective in RA and that this correlates with reduced FoxP3 expression, which is reversed by TNF α blockade [138]. Regulatory T cell function has also been shown to be defective in CIA, an animal model of RA [139] and similar findings were reported in experimental autoimmune encephalomyelitis (EAE) [140], multiple sclerosis [141], and type 1 diabetes [142–144].

On the other hand, there is an opposing body of evidence of a positive effect of TNF in promoting regulatory T cell development. For example, TNF was shown to activate murine regulatory T cells in a TNFR2-dependent manner resulting in proliferation, upregulation of FoxP3 expression, and increased suppressive activity [145]. Furthermore, TNFR2 expression is a hallmark of regulatory T cell and TNFR2-expressing CD4 $^{+}$ FoxP3 $^{+}$ regulatory T cells represent

the maximally suppressive subset of regulatory T cells [146, 147]. TNF blockade was also shown to abrogate LPS-induced expansion of splenic regulatory T cells in vivo [148]. The role of TNFR2 in promoting regulatory T cell responses in EAE is also supported by the observation that TNFR2 on nonhaematopoietic cells is required for regulatory T cell-mediated disease suppression [149]. In addition, TNF blockade was found to prevent the expansion of regulatory T cells in murine psoriasis-like disease [150] and the activity of natural, but not inducible regulatory T cells, was found to be dependent TNF signaling in vivo [151]. A critical role for TNFR2 signalling has also been demonstrated in the induction of human antigen-specific regulatory T cells by tolerogenic dendritic cells [152].

3.7. Adverse Effects of Anti-TNF. Although TNF antagonists are generally judged to be relatively safe, some adverse effects have been observed, including reactivation of latent tuberculosis, which remains the most important safety concern. This may be explained by the results of a recent study showing that TNF blockade inhibited the expression in lymphocytes of two molecules involved in defence against intracellular pathogens: perforin and granulysin [153]. It was also shown that anti-TNF therapy led to a reduction granulysin expressing CD8⁺ T cells with antimicrobial activity against *Mycobacterium tuberculosis*, which helps to explain the increased risk of mycobacterial infection in patients treated with TNF blockers [153].

TNF antagonists have been shown to be very effective for the treatment of various diseases, including psoriasis and it is therefore surprising that new onset or exacerbation of cutaneous psoriasis has been described after initiation of anti-TNF therapy. Although the reasons for this are unknown, a possible clue comes from the finding in mice that TNF blockade exacerbates murine psoriasis-like disease by enhancing the activity of Th17 cells [150]. This will be discussed further in the next section.

3.8. Paradoxical Effects of TNF on Th1/Th17 Responses. Recent findings from our laboratory have shed new light on the complex relationship between TNF and the immune system by the discovery of an endogenous regulatory pathway, triggered by TNFR1, that specifically targets Th1 and Th17 cells [154]. Thus, blockade of TNF, or deletion of TNFR1, was found to cause an expansion of collagen-specific Th1 and Th17 cells in the spleen and lymph nodes of mice with CIA. This was not only due to a diversion of Th1/Th17 cells away from the site of inflammation and into the lymphoid organs because the same phenomenon was observed in collagen-immunised TNFR1^{-/-} (but not TNFR2^{-/-}) mice in the absence of inflammation [154]. Furthermore, a very recent study that reproduces our findings in a model of reactive arthritis documents a global expansion of Th1/Th17 cells in the lymph nodes, spleen, and joints of TNFR1^{-/-} mice [155]. Importantly, we confirmed using an adoptive transfer system that this expanded population of Th1/Th17 cells is highly pathogenic when the anti-TNF “brake” is removed

[154], which may help to explain why disease flares following withdrawal of TNF blocking drugs.

The differentiation and survival of Th1 and Th17 cells are largely controlled by IL-12 and IL-23, respectively, and these two cytokines share a common p40 subunit. We therefore hypothesised that the expansion of Th1 and Th17 cells following blockade of TNF was due to an increased IL-12/IL-23 p40 expression. This was subsequently confirmed in vitro and in vivo at the level of mRNA and protein. In addition, we were able to show that the expansion of Th1 and Th17 cells could be reduced in TNFR1^{-/-} mice by blockade of IL-12/IL-23 p40 [154]. Hence, our data show that at least part of the expansion of Th1 and Th17 cells by TNF inhibition is by augmented p40 expression. The other part of the expansion may be explained by increased trafficking of effector T cells into lymphoid organs. As discussed above, Cope have shown that prolonged exposure of lymphocytes to high levels of TNF in RA leads to a state of T cell hyporesponsiveness due to perturbation of TCR signal transduction pathways [132]. This is fully in agreement with our concept of an immunoregulatory role for TNF.

There is a considerable degree of support in the literature for an inhibitory role for TNF on pathogenic T cell activity. For example, a very early study demonstrated that administration of rTNF to young lupus-prone mice led to protection against lupus and reduced levels of antinuclear antibodies [156]. Comparable findings were reported in murine type 1 diabetes [157] and more recently in adjuvant arthritis [158]. It was also reported that TNF selectively inhibits IL-12/IL-23 p40 expression in human and mouse myeloid cells in vitro, which is in agreement with our research [159, 160].

It is clearly a priority to establish whether anti-TNF therapy has a similar effect in RA to that observed in CIA and one very recent study has confirmed this [161]. In addition, we carried out a longitudinal study of two patients with RA treated with anti-TNF α biological agents in order to assess their Th17/IL-17 levels before and after the start of anti-TNF α therapy. Significant increases in circulating Th17 cells, but not in Th1 cells, were detected in patients after anti-TNF α therapy and this was accompanied by increased production of IL-12/23p40 [162]. There was also evidence of an inverse relationship between baseline Th17 levels and the subsequent response of patients with RA to anti-TNF α therapy. These findings, confirm that anti-TNF α therapy results in an expansion of Th17 responses and a Th17-targeted therapeutic approach may be useful for anti-TNF α nonresponder patients or in combination with anti-TNF α therapy.

4. Conclusions

Research into the pathogenesis of RA has led to the successful development of anti-TNF α therapy and other biological therapies have now entered the clinic, including anti-CD20, anti-IL-6R, and CTLA4-Ig. These new treatments provide exciting opportunities for probing disease pathogenesis, not only in animals but also in humans. Despite the undoubted success of anti-TNF α therapy in controlling RA, it is not

a cure and the long-term goal is to further elucidate the pathogenesis and aetiology of the disease in order to design safer, more effective, and more durable forms of treatment. To achieve this goal, comprehensive analyses of mechanisms of action of biologics entering the clinic will be essential to optimise therapy with the ultimate aim of providing a cure.

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