ANTI-TNF THERAPY

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1. ABSTRACT

TNF is one of the most important cytokines produced by macrophages. TNF is a very important component of host defence, released very rapidly after all types of injuries and stimuli. The kinetics of TNF release are short, and so it is perhaps not surprising that prolonged TNF production is associated with pathology. This was first elucidated in rheumatoid arthritis, but extends to other chronic inflammatory diseases such as Crohn's disease and psoriasis.

In this chapter, the discovery of anti-TNF therapy is reviewed, with its benefit but also its limitations. The potential of anti-TNF therapy in other diseases, e.g. cardiovascular and fibrosis is discussed, as is the opportunity to define ways of blocking TNF synthesis.

2. INTRODUCTION

TNF is a member of the large family of cytokines, not hormones but which are important local signalling molecules transmitting information from one cell to another (1). Different cytokines convey different messages, but cytokines are key players in every important biological process, including immunity, inflammation, cell growth, migration, fibrosis, vascularization etc. So it is not surprising that abnormalities of these key mediators, molecules which are important enough to be conserved through evolution, may be involved in disease processes. What might not have been predicted was that removal of a single upregulated cytokine can make a clinical difference. This is best documented for anti-TNF (2), but is also true for blockade of IL-6, GM-CSF and IL-1. In this review we will summarize

the current state of knowledge about cytokine expression and dysregulation in rheumatoid arthritis (RA) and other diseases and the role of TNF. This topic is appropriate for this book, since the great majority of the TNF is produced by macrophages. The knowledge gained has impacted our understanding and therapy of other diseases also, and by focusing on cytokines, major rate limiting steps and hence therapeutic targets there are opportunities for planning of therapy for many unmet needs.

3. RHEUMATOID ARTHRITIS: MORE THAN JUST A CYTOKINE DISREGULATION

Knowledge about the pathogenesis of rheumatoid arthritis (RA) has augmented in recent years. RA was first defined as an autoimmune disease due to the presence of rheumatoid factor, an autoantibody to the Ig hinge regions, then by its linkage to HLA and to HLA-DR4 especially (3). More recently our knowledge of its autoimmune nature has been greatly amplified by the revelation that the RA autoantigens are post-translational modifications of several abundant proteins, with loss of an amino (NH2) group from arginine, to form 'citrullinated' proteins. The ones which appear to be most important are citrullinated enolase and vimentin but also fibrinogen and collagen type II (4). Some of the auto-antibodies are pathogenic, for example activating osteoclasts (5). There is clear heterogeneity in RA, with the patients expressing HLA-DR4 also producing these antibodies, not RA patients with other HLA-DRs, which are thus 'seronegative'. Both types of RA patients however respond equally well to anti-TNF therapy (3).

Our work on the role of cytokines in RA was initially triggered by two events. First was

exploring an old conundrum of the 70's/80's, was there an immune response to cancer in humans that might be therapeutically useful? In mice it was clear at the time that there was an immune response to cancer, but the murine cancers were 'foreign' as they expressed viral antigens. In contrast immune responses were not detectable in human cancers. One of the authors (Marc Feldmann) had trained scientifically at WEHI, where the legacy of Sir Frank McFarlane Burnet whose 'clonal selection theory' underpinned research in the field of immunity and autoimmunity was still notable. The concept of autoimmunity suggested an approach. What was needed for immunotherapy of cancer was to induce an autoimmune response to it. But this was not so simple, as it was not known how an autoimmune response was induced.

However in the early 1980's there was an increasing amount of data genetically associating different autoimmune diseases with various HLA-DR specificities (6). Subsequently the histological demonstration of upregulated expression of HLA-DR in local sites of autoimmune diseases (e.g. type 1 diabetes, thyroiditis, RA etc) was linked to the role of HLA-DR in antigen presentation (7, 8) by Marc Feldmann and colleagues (Bottazzo, Pujol-Borrell and Hanafusa) who published a new hypothesis in 1983 proposed that locally upregulated HLA-DR augmented antigen presentation of local autoantigens. As self-reactive T cells are present in normal humans (and mice) this might be sufficient to initiate autoimmunity (8), if regulatory pathways were ineffective. Since at the time the only known regulators of HLA-DR expression were cytokines (9), this hypothesis focused attention on the role of cytokines in autoimmune disease. A 'cottage industry' was engendered of expressing cytokines e.g. IFNγ locally in transgenic mice which as predicted triggered autoimmunity in these mice (10).

4. HOW WAS TNF IDENTIFIED AS A THERAPEUTIC TARGET?

The cellular basis of the hypothesis, of upregulated antigen presentation and presence of autoantigen reactive T cells was established using human thyroid autoimmune disease tissue with the able assistance of Marco Londei (11, 12). But determining which cytokines were of importance was not possible in thyroid tissue as it was rendered quiescent before surgery. However in rheumatoid arthritis, a much more important unmet need with poor prognosis, it was possible to sample active disease tissue and explore cytokine production (13).

How could cytokines be evaluated in disease tissue? Fortunately, the advent of the molecular biology revolution with the cloning of cDNAs for cytokines provided the necessary research tools for this work in rheumatoid arthritis (14). Our approach was to evaluate which cytokines were produced at site of the disease, and RA is one of the most accessible autoimmune diseases. Glenn Buchan was the post-doctoral fellow who first succeeded in modifying the conventional techniques to be useful for small human disease tissue samples and we chose to measure mRNA as a close reflection of local cytokine synthesis. We were surprised to find that all the RA synovial samples produced all the cytokines which we could measure (15, 16). At the same time other groups were also investigating cytokine production in joints. Gordon Duff's group detected IL-1 and TNF (17), Jean-Michel Dayer (18) Firestein and Zvaifler (19) were also active in this field. Since in health, cytokine production, at least for immune/inflammatory cytokines, is transient, detecting them in all samples was abnormal, and was considered to reflect long term production. This was not due to long

half-life of mRNA, so it was due to continued stimulation of production. This result was exciting as it validated that in RA there was cytokine disregulation, but from the therapeutic perspective, the important question remained, which cytokine might be a therapeutic target (20)? This was relevant as the biotech industry had developed the capacity to generate specific anti-cytokine inhibitors, monoclonal antibodies or antibody like receptor Ig fusion proteins. Which single cytokine to target when there are many present with similar actions was a major dilemma, and led to many of our competitors in this field deciding, on the basis of 'cytokine redundancy' that cytokines were not good therapeutic targets. Fionula Brennan was our post-doctoral fellow in 1988/89 who resolved this dilemma, by analysing the regulation of cytokine expression in a dissociated synovial cell culture model of arthritis, which we had developed.

Prior to Fionula's publications, culture of RA synovial cells was simple and serial passage only kept the synovial fibroblasts alive. The inflammatory/ immune cells, which are the great majority of an RA sample died or were discarded. Marc Feldmann's PhD at WEHI began in 1969 by learning how to optimise the *in vitro* immune response of mouse spleen cells (21). So the challenge of maintaining the great majority of immune/inflammatory cells from synovium alive to study cytokine dysregulation and this attempt to define a therapeutic target was not beyond our skill set. By modifying conditions (serum type and amount, oxygenation, cell density) cultures sustaining mixed synovial cell function for 5-6 days were established and used to analyse synovial cytokine dysregulation. Fionula Brennan obtained the paradoxical result that blocking a single cytokine, TNF, totally abrogated IL-1 production within 2-3 days in 7 consecutive RA operative samples (16).

This led to a new concept of the 'TNF dependent cytokine cascade'. As is often the case, work from other directions led to a similar conclusion. Tony Cerami and his colleagues were studying bacterial sepsis in mice, and showed that the first cytokine detectable in mouse blood was TNF within 30 mins then IL-1 and later still IL-6 (22). As had been found in synovial cultures, neutralization of TNF by anti-TNF inhibited the production of IL-1 (Fong et al. JEM 1989). This suggested that the TNF dependent cytokine cascade could also operate *in vivo* in mice.

The unexpected 'TNF dependent cytokine cascade' concept was tested in synovial cultures for all the pro-inflammatory cytokines, produced in RA synovial cultures, which could be evaluated. TNF blockade of synovial cultures rapidly down-regulated GM-CSF (23), IL-6, IL-8 (24) and others, later it was also found to also apply to anti-inflammatory mediators IL-10, IL-1 receptor antiagonist, soluble TNF-Receptor. The TNF dependent cascade clearly showed that TNF blockade was different to other cytokine blockade, as IL-1 blockade did not diminish TNF. This suggested that TNF might be the elusive therapeutic target for RA.

At the time (1989) the predictive capacity of animal models of disease was considered poor, for example, collagen induced arthritis had been used to predict that killing CD4 T cells would be therapeutic for RA, which it wasn't, and little has changed in that context. So testing anti-TNF in animal models of arthritis only was not a high priority and only happened after the key human synovial experiments had been completed, once suitable anti-murine TNF antibodies had been generously donated to us by Dr Robert Schrieber, Richard Williams was able to show its ameliorative, but not curative effects of anti-TNF given after disease onset in mice with collagen induced arthritis (25).

5. HOW DID ANTI-TNF BECOME STATE-OF-THE-ART THERAPY OF RA?

With a presumed rationale for anti-TNF therapy in RA, the next challenge was to test this in patients. It was fortunate that specific TNF inhibitors had been generated by several groups in the biotech industry, based on Tony Cerami's influential concept that TNF was the driver of bacterial septic shock, which if blocked could save hundreds of thousands of lives (26). But our ideas of the role of TNF in RA at the time (1989 onwards) were not widely accepted and the response of several companies that had produced suitable inhibitors who we talked to at length was eventually negative. But when James Woody, a former colleague joined Centocor, a monoclonal antibody focused company as their chief scientist, we found an ally. He was convinced that Centocor should work with us, and as his clinical colleagues were at the time preoccupied with testing anti-CD4 antibody in rheumatoid arthritis, he and a few helpers worked with Ravinder Maini and Marc Feldmann to test our concept clinically. They initially provided a modest grant ($100,000) and the necessary amount of chimaeric anti-TNF antibody, which was then in short supply, then known as cA2, now infliximab (Remicade®) to test in 10 patients. Not a lot for an academic proof of principle, but enough. No dose response could be done, so we chose the highest dose which had been safely evaluated for sepsis, 10 mg/Kg, coincidently the same dose as another anti-TNF antibody that we had used in mouse experiments.

The results were dramatic, and the first patients reported relief from fatigue, as if a load had lifted, even while the slow (3 hour) infusion still ongoing (27). The enthusiasm of the

patients in an open-label trial and their hope for a good outcome argues for caution in interpreting results, but the unprecedented degree of rapid clinical change, coupled to marked changes in blood tests within days, in many cells (monocytes, granulocytes) and inflammatory proteins (IL-6, CRP) suggested real clinical benefit. Despite the initial great benefit, all patients relapsed between 12-18 weeks, and as the first patients desperately wanted more such treatment, approval was sought from the hospital Ethical Review Board to re-treat them, as they relapsed which was granted. This was a key experiment, as it begins to address an important question, whether if TNF is blocked, another 'redundant' pro-inflammatory cytokine pathway would take over? Retreatment re-induced the same degree of benefit in 7 patients, but the duration of benefit 'tended' to get shorter (28). The latter was not a scientific conclusion, given the limited number of patients involved (8) and also the halving of the therapeutic dose of cA2 administered.

From the successful proof-of-principle described above, a formal double blind, randomized, placebo controlled trial was performed comparing 1 and 10 mg/ml cA2, and a placebo (human serum albumin, with same appearance), as a single infusion, assessed at 4 weeks. The short treatment duration was because a major benefit had been noted within that period with 10 mg/Kg, and since patients had been taken off all 'disease modifying drugs', too long a duration would have led to 'drop-outs' especially in the placebo controls, with loss of statistical power and thus a major risk of a failed clinical trial (29).

Both doses were effective as compared to placebo, and this together with the 'open' retreatment study opened the path to longer term studies. The key trial was to define the optimal 'unmet need' indication for anti-TNF therapy. As an important unmet need was

treatment for MTX failures, this was the population treated. Unusually we chose to continue MTX therapy despite its inadequate benefit, and so this was a form of 'adjunctive' or combination therapy. To permit easier evaluation and promote safety, the MTX dose was standardised at the very low end, 7.5 mg/week, and 3 doses of anti-TNF used 1, 3, 10 mg/Kg at monthly intervals. Again the results were very interesting. MTX even at the very low dose in the 'MTX failures' augmented the response after administration of anti-TNF notably, dramatically at 1mg/Kg cA2, but also at the higher doses (3 and 10 mg/Kg) (30).

From this trial has sprung the major use of all the anti-TNFs in combination with MTX, and also successful 'use' patents held by the Kennedy Trust for Rheumatology Research, which have gained royalties, permitting amongst other things the relocation and rebuilding of the Kennedy Institute of Rheumatology to the University of Oxford.

Based on the trials of cA2 (now termed infliximab) with MTX, all the other anti-TNFs, were tested in combination with MTX and the positive results in each case have led to its routine use in combination therapy (31, 32).

6. MECHANISM OF ACTION STUDIES

While some Pharmaceutical companies think that if a medicine works clinically the mechanism of action is irrelevant, academics are very curious and keen to understand why a medicine actually works. This is because successful therapy is a very powerful probe of human biology, one of few available. Hence, there have been many studies probing anti-

TNF therapy during its early academic led development, which regrettably were curtailed in Phase 3 as it became clear anti-TNF was going to be going on sale.

From the first proof of principle studies, changes in inflammatory proteins and blood cellular counts were noted, 'longitudinally', using the same patient's prior samples as a control. More informative was the first randomized study, as it had both placebo dose response and longitudinal comparisons. Much of what we know mechanistically was first elucidated then. Very painfully, the samples from a subsequent longer term study defrosted in a freezer disaster.

Rapid changes in inflammatory proteins, within a few hours in IL-6 for example confirm that TNF has direct effects on production of other cytokines, including chemokines, and that the 'TNF dependent cytokine cascade' defined in synovial culture experiments operates in RA *in vivo* (29). Other inflammatory protein levels, including CRP, and SAA fall in a few days. Inflammatory cell numbers, granulocytes and monocytes, elevated in active disease, rapidly reduce as do platelets, while T lymphocytes, relatively low in active RA increase rapidly. So rapidly (hours), that the only possible plausible mechanism is egress from joints, probably due to reduced adhesion molecule expression (33).

Immunohistology has certain problems due to potential sampling errors due to very small samples, problems in the timing of biopsy, and lack of quantitation of changes by the techniques of 1990's. But certain changes are so dramatic as to be unequivocal, such as the reduced cellularity, both of mononuclear and lymphoid cells. The mechanism of reduced cellularity is not fully understood, reduced ingress, augmented exit are documented in other

studies, increase in apoptosis remains controversial. Other important findings were normalization of haematological abnormalities (vide supra) including reduction in fibrinogen and platelets, presumably reducing the risk of thrombosis and of cardiovascular disease. Abnormal immunity is normalized, the low response to exogenous antigen of blood cells and by skin test is augmented within a week. Tregs become normalized. Markers of probable tissue damage are reduced e.g. levels of MMPs, and of cleavage products of connective tissue. VEGF is partly reduced, suggesting angiogenesis maybe partly reduced, which was confirmed in histology studies (reviewed 34, 35).

From the retreatment studies which were performed on the first cohort of patients, when they had relapsed, it was clear that the reinduced benefit, while similar in magnitude had shorter duration. This led to investigation of the immunogenicity of the anti-TNF antibody. There is an extensive literature that all proteins, even autologous ones are immunogenic, from the time of Jacques Oudin and Rodkey that anti-idiotype antibodies to reinjected self immunoblogulin in rabbits (36).

Interferons which upregulate antigen presentation are immunogenic in most patients, limiting the duration of their usefulness. In contrast there is an extensive literature from the 1960's and 1970's that while aggregated Ig administered subcutaneously was immunogenic, deaggreagated Ig injected intravenously is non-immunogenic, and Weigle, Dresser, Mitchison, Basten and others described the induction of Immunological tolerance by deaggreagated Ig at either very low or very high doses (37, 38) termed 'Low dose' or 'High zone' tolerance. Therapeutic antibodies are rigorously deaggregated, or else there would be toxic side effects.

It was found that intravenous Infliximab (the name now used for the antibody we used in the clinical trials) was tolerogenic at high dose. At 1mg/kg, anti-Infliximab antibody was detected in about half of patients, but only about an eighth at 10mg/kg, in keeping with that concept. In the presence of low dose methotrexate, anti-Infliximab antibodies were further reduced (39).

While the presence of anti-therapeutic antibodies does not interfere with response short term, long term there is evidence of better outcomes in patients that do not form antibodies (40). Currently there is little evidence that utility and sales are restricted by immunogenicity, currently antibodies form half of the world's top 10 best-selling medicines, with anti-TNF the best selling drug class.

7. LIMITATIONS OF ANTI-TNF THERAPY

While there is excitement and a sense of achievement of initiating a new therapy, upon closer examination, with time, there are always limitations which emerge. And while patients are initially mostly greatly relieved, with time there is realization that more is needed. The patients appreciate progress, but they would like to get closer to a cure. What are the residual problems after anti-TNF therapy? The most important symptom is residual pain, varying greatly between patients, many have persistent fatigue and tenderness. A major problem, joint damage is markedly reduced, cartilage damage less so than bone damage (34).

As might be expected from blocking TNF a major host defence mediator, there are increases in some infections. First noted is infection with intracellular organisms, such as TB, less often with listeria etc. Regrettably with reduced inflammation the symptoms of TB are different, and the first patient with recrudescence of TB died as it was diagnosed late. But with effective screening the risk of recrudescence of TB falls from about 1/2000 to 1/20,000 (41) and does not limit its use in the Western world. Other infections change a bit, there is more skin infection. But in the big post-marketing registers such as the UK, the overall risk of infection does not change compared to severe RA treated by other means (42, 43). This is probably due to a combination of two reasons, first RA patient's immune function is compromised by the disease itself, secondly anti-TNF does not block all of TNF signalling. It is very unlikely that most of TNF, a local mediator, can be effectively blocked from signalling to an adjacent cell in direct contact, by an antibody. This limited inhibition probably reduces TNF levels from the elevated levels to normal, but is not a total blockade which intracellular TNF synthesis inhibitors could do.

With time the percentage of patients responding, initially 70% reduces, and the degree of response can also diminish. This has opened up great debate and interest as to what is the best treatment for anti-TNF low responders (44).

8. WHAT MIGHT BE DONE TO GET CLOSER TO A CURE? COMBINATIONS

Sale of the anti-TNF medicines (5 on market) dwarf those of other new drugs for RA. Thus it appears that the clinicians prescribing new treatment for RA considered its efficacy and safety, and familiarity with how to use anti-TNFs superior to its competitors. This may be

partly helped by regulatory and purchaser priorities. But it is worth mentioning that in the 20 years since anti-TNF clinical trials first reported success, a multitude of new medicines have been tested and those that completed trials and come to market have roughly comparable efficacy. It does not seem that in a complex heterogeneous disease with multiple pathways deregulated, that a single drug could yield a cure (45). That prediction may be wrong, but is reasonable on the basis of evidence available today.

So what could be the future? Looking sideways i.e. comparison, has a long tradition in science. In two fields HIV and haematological malignancies it is clear that multiple drugs, combination therapy has dramatically changed the prognosis, for HIV from certain death (46), to life with some niggling side effects of the drugs.

So what combinations might get many patients closer to a cure? There have been attempts to augment the effect of one anti-TNF agent etanercept (TNFR2 Ig fusion protein) by combination with anakinra (IL-Ira) (47) or abatacept (CTLA4 Ig) (48). These trials were smallish but there was no increase in efficacy at all, but a marked increase in infections. What might be learnt from this? Probably that depressing TL inflammatory function (e.g. with etanercept and anakinra) too much is not helpful. It should be noted that the 'redundancy', the similarity between effects of TNF and IL-1 on their targets is probably about 90%. Combination of etanercept, with some inhibition of host defence, with abatacept, which is designed to block the initial antigen presentation between T lymphocytes and APC at the beginning of an immune response (e.g. to infectious organisms) is also risky, with a degree of hindsight. Of course these conclusions might depend on dose or spacing between administration, but it is now unlikely that these combinations might be

revisited.

But more promising might be combination of anti-TNF therapy with medicines that target mechanisms that maintain disease chronicity but do not interfere with host defence against infection. The mechanisms that comes to mind are blocking angiogenesis, and blocking the fibroblast like synoviocytes (FLS) that erode into cartilage. There is evidence to support these concepts that blocking angiogenesis (34, 35) and FLS (49) might be effective, including some combinations in an animal model, collagen induced arthritis (50). But it needs a concerted effort to define the best combination to add to existing therapy. The most likely existing therapy would be anti-TNF, as the biggest unmet need is how to enhance low responsiveness to anti-TNF. This is discussed in detail elsewhere (45).

9. FUTURE ANTI-TNF AGENTS

In parallel to finding ways to enhance low responsiveness to anti-TNF therapy, further research into the molecular mechanisms that govern inflammatory response may lead to identification of new targets for specific and ideally orally administrated therapeutic intervention.

Since macrophages are the main producers of TNF (51), one approach to new target identification could consist of mapping the molecular pathways and signalling events that lead to TNF production in macrophages. Macrophages are considered to be of central importance in the pathogenesis of RA. The increase in the number of sublining macrophages is an early hallmark of active rheumatic disease (52), with the high numbers of

macrophages being a prominent feature of inflammatory lesions (53). The degree of synovial macrophage infiltration correlates with the degree of joint erosion (54) and their depletion from inflamed tissue has a profound therapeutic benefit (55). Due to the major role of macrophages in RA, the effect of anti-rheumatic treatment on macrophage numbers, activation and function is considered to be an objective readout of their efficacy (56).

Macrophages are heterogeneous, with many subpopulations now known. They also demonstrate remarkable plasticity that allows them to efficiently respond to environmental signals and change their phenotype and their physiology in response to cytokines and microbial signals. For example, colony stimulating factors (CSFs) play a key role in macrophage polarization. Macrophage CSF (M-CSF) is constitutively produced by host tissue cells, such as fibroblasts, stromal cells, osteoblasts, even in the absence of inflammation and has a largely homeostatic and resolving role, whereas granulocyte-macrophage CSF (GM-CSF), is produced by the same cells during inflammation and has a clear pro-inflammatory effect on macrophages (57). These changes can give rise to populations of macrophages with diverse functions, which are phenotypically characterised by production of pro-inflammatory and anti-inflammatory mediators (58).

Although the extent of heterogeneity of macrophages in RA have not been fully uncovered, there believed to be a mix of pro-inflammatory infiltrating macrophages and less inflammatory tissue resident ones. Efforts to understand the polarization of macrophages and how it influences disease progression, have led to identification of novel signalling pathways and strategies that target components of these pathways with varied specificity and selectivity. For example, blockade of GM-CSF inhibits the development of arthritis (59).

Since depletion of GM-CSF is effective, several therapies are in phase 1 or phase 2 clinical trials or preclinical studies using GM-CSF-specific antibodies, GM-CSF receptor-specific antibodies.

Our recent work has indicated that GM-CSF (along with IFN-γ) is a major inducer of interferon regulatory factor 5 (IRF5), a transcription factor defining pro-inflammatory macrophage polarization (60). IRF5 is involved in the positive regulation of Th1/Th17 associated mediators, such as IL-1, IL12, IL-23 and TNFα (60). We have reported that IRF5 forms a protein complex with NF-κB RelA to drive a sustained induction of the human TNF gene (61), and lately extended this observation to demonstrating that interactions of IRF5 with RelA is a common mechanism of pro-inflammatory gene regulation by IRF5 (62).

IRF5 is a member of a family of transcription factors, originally implicated in anti-viral response and interferon (IFN) production (63). Subsequent studies revealed their multifaceted role in regulation of anti-microbial responses and cell differentiation (63). In murine model of antigen-induced arthritis (AIA) synovial macrophages are characterised by high levels of IRF5 (64). IRF5 is also a genetic risk factor for many autoimmune diseases, including RA where it was identified as a new risk locus by the genome-wide association study (65) and contributed to the modulation of the erosive phenotype (66).

Considering the key role of macrophages in synovial inflammation, it is tempting to speculate that IRF5 expression is a finely tuned balance between macrophage adaptive versus pathological responses and thus IRF5 represents a new post anti-TNF target for therapeutic intervention. In fact, recent study reported that silencing IRF5 in infarct

macrophages resulted in reprogramming of macrophage phenotype, resolution of inflammation and improved infarct healing (67). How blockade of the IRF5-RelA interaction would compare with TNF blockade in terms of efficacy and safely is not currently understood.

10. OTHER INDICATIONS

(a) Cardiovascular

One of the first indirect evidence of cytokine production in vascular disease was the upregulation of HLA-DR in human atherosclerotic lesions by smooth muscle cells (68). Tumour necrosis factor alpha was the first cytokine to be identified in the human atherosclerotic plaque (69) shortly thereafter. TNFα has a significant role in the activation of the endothelium (70) and in the upregulation of adhesion molecules, a key event in the first steps of atherosclerotic disease.

TNFα is involved in multiple actions on different cell types in the lesion such as adhesion molecule expression and foam cell formation. Blocking TNFα by TNF binding protein or IL-1 by IL-1 receptor antagonist partially protected apoE KO mice from atherosclerosis (71). Myeloid cell production of TNFα was the most relevant to atherogenesis. In mice on a high fat diet, the plaque area in apoE-/- TNF-/- mice was 50% smaller than in apoE-/- mice (72). Branen, *et al.* showed that transplantation of bone marrow from apoE-/- TNF-/- into apoE-/- mice resulted in an impressive 83% reduction in lesion size after 25 weeks. In the same study, treatment of apoE-/- mice with the TNF blocker recombinant soluble p55 also led to a reduction in lesion size indicating that TNFalpha plays an important role in atherosclerosis. Similarly, Ohta, *et al.* showed a slightly smaller decrease in lesion size in apoE-/- TNF-/- mice

on a normal chow diet (73). In this model, expression levels of adhesion molecules ICAM-1 and VCAM-1, and chemokine MCP-1 (CCL-2) were also significantly decreased, along with reduced scavenger receptor expression and uptake of oxLDL in double knockout mice.

Peripheral blood levels of TNFα also predict the development of myocardial infarction (Ridker) in patients with known CVD. NFκB is an important pathway leading to the production of TNFα in human atherosclerotic plaques (74). However, TNF blockade has never been tested properly in CVD after the failure of TNF blockade in heart failure patients (75-77) leading to failed opportunities for use of biologics in CVD.

(b) Acute injuries and Fibrosis

Macrophages undoubtedly have an important role in wound healing. They have been shown to be key in orchestrating fracture healing (78). They also play a crucial role in cutaneous wound healing, their early deletion leading to impaired granulation tissue formation whilst in the mid-phase of healing depletion resulted in severe wound haemorrhage (79). Macrophage subtypes are in part defined by the types of cytokines they produce. For example, the M2 phenotype is characterised by the expression of TGF-β whilst the classically activated M1 cells secrete pro-inflammatory cytokines. It has become accepted that during the early phases of healing classically activated M1 macrophages predominate whilst the repair phase is dominated by cells with the alternatively activated M2 phenotype (80). Subsets within this grouping have been ascribed specific functions, for example M2a pro-fibrotic macrophages in cutaneous wound healing (80). However, it is not clear whether classifications based on *in vitro* phenotypes are representative of what

happens *in vivo*. In particular M2a macrophages may not necessarily predominate *in vivo* during tissue repair (81).

The emphasis on the accepted roles of certain cytokines produced by subsets of immune cells has formed the basis of numerous experimental studies (82). An example of this that inflammation driven by TNF tends to be detrimental and the archetypal profibrotic cytokine is TGF-β. Fibrosis is especially difficult to study as primary human tissues, especially at early stages of the disease, are difficult to access and animal models are of necessity based on toxic injuries that are rarely encountered in human disease. Whilst data from these models have highlighted the importance of TGF-β, all late phase clinical trials to date of TGF-β1 inhibition have failed, with some reporting significant adverse effects (83, 84). The cell responsible for both the matrix deposition and contraction in all fibrotic diseases is the myofibroblast. Our studies of Dupuytren's disease, a common fibrotic condition of the palm of the hand that affects approximately 4% of the general UK and US populations, were based exclusively on human samples from patients with relatively early stage disease. In addition to myofibroblasts, we found significant numbers of immune cells, including classically and alternatively activated macrophages. Utilising a system similar to the one previously used to identify TNF as a therapeutic target in rheumatoid arthritis, we determined the cytokines secreted by freshly disaggregated cells from Dupuytren's nodules. We then examined the effects of these on myofibroblasts precursor cells from patients with Dupuytren's disease compared to normal fibroblasts from the same individuals and from healthy donors. Exogenous addition of TNF, but not other cytokines, including IL-6 and IL-1β, promoted differentiation specifically of palmar dermal fibroblasts from Dupuytren's patients into myofibroblasts. A previous GWAS study identified the role of Wnt in

Dupuytren's disease (85). We demonstrated that TNF acts via the Wnt signaling pathway to drive contraction and profibrotic signaling in these cells. Neutralising antibodies to TNF inhibited the contractile activity of myofibroblasts derived from Dupuytren's patients, reduced their expression of α-smooth muscle actin, and mediated disassembly of the contractile apparatus (86). These data form the basis of ongoing clinical trials funded by the Wellcome Trust and the UK Department of Health to assess the efficacy of local administration of anti-TNF directly into the nodules of patients with early Dupuytren's disease.

The importance of studying tissues from the early stages of the disease is emphasized by accumulating evidence that inflammation precedes almost all fibrotic processes (87). We found that freshly disaggregated cells form Dupuytren's disease secreted TNF at the levels that were optimal for promoting the differentiation of palmar fibroblasts from affected patients into myofibroblasts. At passage 2 TNF levels were negligible whilst levels of TGF-β1 increased three fold through autocrine production by myofibroblasts (86). Importantly, the levels of TNF secreted *in vitro* were unaffected by the total number of primary disaggregated cells over almost a log range, indicating considerable auto-regulation. Our findings emphasise the importance of studying primary human diseased tissues (88) and comparison with appropriate controls, and may go some way towards explaining the failure of late phase clinical trials targeting TGF-β1.

The early macrophage response following injury is typically described as infiltration by the M1 subset and related to clearance of microbes and tissue debris, whilst the secondary M2 response is usually considered to orchestrate tissue repair (82). However, this is probably

somewhat simplistic and emerging evidence suggests that whilst persistent or high levels of inflammation are detrimental to tissue healing, low levels of pro-inflammatory cytokines can initiate downstream healing responses. Again using primary human tissues, we found that supernatants derived from fractured bone fragments but not from surgically cut bone promoted the chemotaxis and osteogenic differentiation of human mesenchymal stromal cells (MSC). The cytokine primarily responsible was TNF, which promoted MSC chemotaxis at 1pg/ml and osteogenic differentiation of MSC at 1ng/ml. Pre-incubation of MSC with TNF also enhanced the effects of other chemokines (89). Having elucidated a target utilising representative primary human samples, we went on to investigate the mechanism using a murine model. Local administration of TNF only within the first 24h post injury enhanced fracture healing whilst anti-TNF and IL-10 impaired healing. Addition of exogenous addition TNF enhanced recruitment of neutrophils and monocytes through CCL2 production whilst depletion of neutrophils or inhibition of CCR2 resulted in significantly impaired fracture healing (90). Local administration of TNF at the fracture site in osteoporotic mice improved healing during the early phase of fracture repair.

Our data would suggest that TNF can modulate healing in a variety of ways, ranging from initiating the events culminating in fracture healing through to driving fibrosis. The levels of this cytokine as well as the duration of secretion appear to be important in determining tissue response.

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