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REVIEW ARTICLE

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Exploring the signalling pathways promoting T cell effector responses in chronic inflammation

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Abstract Over the last decade, it has become clear that T helper cell differentiation is determined by a programme of gene transcription, which is in turn dictated by signalling pathways emanating from both T cell antigen receptors and cytokine receptors. This model has provided an experimental framework for exploring the molecular mechanisms through which T cell effector responses initiate autoimmunity, chronic inflammatory disease, and allergy. Much less clear are the processes that regulate T helper cell differentiation and effector responses in established chronic inflammatory diseases such as rheumatoid arthritis. This review describes recent experimental data which suggest that the inflammatory process profoundly influences T cell receptor and cytokine signal transduction pathways in such a way as to attenuate both immunoregulatory and host defence mechanisms on the one hand, while promoting cell survival and effector responses on the other. These findings are consistent with a model in which the inflammatory response is initiated primarily by antigen-driven T cell effector responses, while the chronic phase of the disease process is sustained by cytokine-driven effector responses.

Key words Cytokines · Inflammation · T cell activation · Tumour necrosis factor alpha (TNF)

There exists a reciprocal relationship between immunity and inflammation. On the one hand, it is recognised that the adaptive immune system plays a central role in coordinating inflammatory responses (reviewed by Mosmann and Coffman[1]). On the other hand, it has become appreciated that the inflammatory environment profoundly influences adaptive immunity, not only at the level of T cells,[2] but also at the level of antigen-presenting cells such as dendritic cells (DC).[3] Exploring how T cell effector responses are maintained and how these responses perpetuate the inflammatory process is important for understanding the role of T cells in the pathogenesis of diseases such as rheumatoid arthritis. This is especially important for a disease in which the associations between MHC class II haplotypes and disease susceptibility or severity implicate CD4+ T cells directly in the effector process.[4]

Recent evidence suggests that there exists a coordinated programme of molecular events during T cell differentiation that leads to the generation of CD4+ Th effector T lymphocytes.[5] This is best illustrated in the context of host immunity to foreign pathogens such as *Listeria monocytogenes* infection or parasitic infestation. Three phases are recognised: an initiation phase, a commitment phase, and a phase of acute gene transcription (reviewed by Avni and Rao[5]). During initiation, naïve T cells are engaged by MHC/peptide complexes expressed on the surface of DC. Only those T cells which form a functional immunological synapse are likely to differentiate.[6] At this point, intracellular signalling pathways emanating from stable clusters of TCR/CD3 complexes are integrated with those from cytokine receptors following stimulation with cytokines derived from innate immune responses, including IL-12 and IL-18. In the case of polarised Th cells, these cytokines activate families of transcription factors called "signal transducer and activator of transcription" or STATs, such as STAT4 for IL-12 (Th1), or STAT6 for IL-4 (Th2).[7] The initial engagement phase may last from a few hours to several days, and in general will result in the production of IL-2, yet despite optimal stimulation by antigenic peptide and cytokines, surprisingly few T cells progress to subsequent phases of maturation.

The commitment phase is characterised by the induction and recruitment of Th subset-specific transcription factors. Examples include GATA3 and c-Maf for Th2 cells, and T-bet for Th1 T cells.[5,7] Once these factors are induced, differentiation is stabilized even in the absence of further TCR stimulation. The third phase, that of acute gene transcription, is determined by secondary contact with antigen and

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necessitates the recruitment of NFAT to the transcriptosome. This transcriptional programme is thought to be monoallelic and stochastic, dictated by both nucleosome disassembly and chromatin accessibility. Thus, specific loci become transcriptionally active through a series of changes to chromatin structure, including chromatin decondensation and remodeling, and recruitment of complexes to the nuclear matrix leading to the formation of additional DNase hypersensitive sites. These sites are markers of stable differentiated T cells.[5] Coincident with these molecular changes in the nucleus are post-translational changes, including histone acetylation, phosphorylation, and demethylation,[8,9] which occur during the S phase of the cell cycle.[10]

While these molecular events go some way to explaining why some CD4+ T cells differentiate into Th1 cell subsets and produce IFNγ, while others are destined to become Th2 cells producing IL-4, IL-5, and IL-13, much less is known about the events which regulate effector responses in the context of established chronic inflammatory responses. In fact, T cells from sites of inflammation, such as the rheumatoid joint, rarely exhibit the characteristics of polarised Th cell subsets.[11] Furthermore, there is much evidence that synovial joint T cells from patients with active RA are hyporesponsive to TCR/CD3 ligation but not to ligation of cytokine receptors,[12] suggesting that there may exist an imbalance between signals emanating from the TCR and cytokine receptors in T cells at sites of inflammation. Such an imbalance in signals could have profound effects on the programme of chronic gene transcription in these T cells (see Fig. 1).

What are the signals that maintain synovial T cells in the active state, and is the programme of chronic gene transcription distinct from that observed during the initial phases of T cell activation and differentiation? Some decades ago it was recognised that RA synovial T cells were activated in terms of cell surface expression of CD69 and HLA class II when compared with their peripheral blood counterparts,[13] produced little in the way of cytokines, and proliferated very poorly in vitro in response to either mitogen, antigen, or CD3 ligation with specific agonistic monoclonal antibodies.[12,14] Proliferative hyporesponsiveness could be reversed to some extent through the addition of exogenous T cell growth factors such as IL-2.[15] This T cell phenotype has been described as "anergic," which is a rather inappropriate term to describe the state of T cells in a disease thought to be T cell-driven. The concept of anergic synovial T cells is also incompatible with the histopathological features of severely inflamed joints which are characterized by follicular lymphoid-like structures resembling germinal centres of lymphoid organs, and suggestive of chronic immune activation.[16]

Over the last few years, models of the function of synovial T cells have been substantially revised in order to account for the existence in inflamed joints of stromal factors which favour T cell survival,[17] an imbalance of pro- and anti-inflammatory cytokines with inflammatory macrophage products predominating,[11] and telomere shortening suggestive of replicative senescence.[18] More recent technological advances, such as analysis of intracellular cytokine expression, have documented a very low frequency of IFNγ- and IL-10-producing synovial T cells,[19,20] as well as defects in Th2 differentiation of populations of purified peripheral blood T cells.[21] These data provide strong evidence for impaired immunoregulatory networks, and indicate that there may be a fundamentally different programme of gene transcription driving effector T cell responses in RA joints. The lack of expression of the costimulatory molecule CD28 on an enriched subset of CD4+ T cells in joints could also contribute to these differences.[22]

Almost a decade ago, we began to address the problem of how the chronic inflammatory process influenced T cell autoreactivity and effector responses, with the ultimate goal of a better understanding of the pathogenic processes involved in RA. At that time, we made two broad assumptions. First, we assumed that the environment generated in chronically inflamed joints would be different to that provided by an acute inflammatory or infectious episode. The second assumption was that chronic exposure to inflammatory cytokines would have different effects to those induced following short-term exposure. Our experimental approach was based on a series of observations arising from a larger programme of work in this laboratory, as well as in other laboratories, which sought to document in depth the broad range of cytokines expressed in rheumatoid joints. The observations of particular relevance included the finding that (1) tumour necrosis factor alpha (TNF) bioactivity was found to persist in synovial joints,[23] (2) both high-affinity TNF receptors (TNF-R) p55 and p75 TNF-R were upregulated on synovial joint T cells,[24] (3) TNF-R were expressed in lymphoid aggregates and colocalised with ligand,[25] and (4) expression of the naturally occuring TNF inhibitors, the soluble TNF-R, were also increased in synovial fluid, but were clearly insufficient to completely neutralize the bioactive TNF in vivo.[26] The implication of these findings was that synovial mononuclear cell infiltrates, including T cells, were chronically exposed to TNF in vivo.

Studies published at that time had suggested that TNF was a growth factor for T cells,[27] suggesting that TNF might promote T cell autoreactivity in vivo. Accordingly, we set out to test this hypothesis using an in vitro culture system which attempted to mimic chronic TNF exposure in vivo, and which was not dissimilar to in vitro models used by many laboratories to explore the effects of cytokines such as IFNγ, IL-4, and IL-12 on T cell differentiation. The results were surprising in several respects. Rather than enhancing T cell growth, prolonged exposure of T cells to TNF induced nondeletional and reversible T cell hyporesponsiveness following stimulation through the T cell receptor (TCR)/CD3 complex.[28] In subsequent studies using a TCR transgenic model, it was possible to demonstrate that these immunosuppressive effects could be reproduced in T cells regardless of their Th phenotype.[29] Thus, peptide-specific production of Th1 cytokines was attenuated by TNF in cultures of T cells derived from B10.D2 mouse strain, while production of Th2 cytokines was suppressed to a similar extent in T cells from Balb/c mice.[29] Significantly, chronic

TNF blockade with neutralising mAb had the reverse effects, enhancing T cell proliferative and cytokine responses. Furthermore, repeated injections of otherwise healthy transgenic mice with anti-TNF also enhanced T cell responsiveness, implying that physiological concentrations of TNF in vivo could regulate T cell activation.[29] We now know from our more recently published studies that this most likely reflects the capacity for chronic TNF stimulation to significantly increase the threshold of T cell activation, such that TNF-treated T cells require stimulation with more antigenic peptide for longer periods of time to commit to activation and IL-2 production.[30]

These results were surprising, but were validated by subsequent studies in mice and humans (reviewed by Cope[2]). First, we demonstrated in 1994 that peripheral blood T cell responses from patients with RA were dramatically restored following treatment with anti-TNF (infliximab, Remicade),[28] a finding reported subsequently by several other laboratories.[31–33] More recently, extensive work in mice deficient for TNF or TNF-R has confirmed that TNF has potent immunomodulatory effects in vivo, and is capable of regulating T cell autoreactivity and autoimmunity when studied in autoimmune-susceptible strains of mice.[2] Indeed, these results were consistent with the studies of Jacob and McDevitt, as well as those of Gordon and Wofsy, who first demonstrated the disease-protecting effects of TNF therapy in NZB/W F1 lupus-prone mice (reviewed by Cope[2]). Similar effects were reported in murine models of type I diabetes. Using a mouse model of multiple sclerosis (MS), Kollias and co-workers[34] have recently demonstrated that while acute TNF exposure is important for T cell priming, chronic TNF is required for the resolution of T cell reactivity to myelin antigens. Sustained T cell reactivity to myelin basic protein (MBP) or myelin oligodendrocyte glycoprotein (MOG) in TNF-deficient mice of the H-2^b strain, which are normally resistant to experimental autoimmune encephalomyelitis (EAE), led to a chronic demyelinating disease. These data could explain the recently reported finding of demyelinating syndromes in a small subset of RA patients treated with a p55 TNF-R-Ig fusion protein, as well as the disease-exacerbating effects of treatment with anti-TNF in patients with MS.[35]

The potent immunomodulatory effects of prolonged TNF exposure has prompted us to explore the molecular mechanisms in more depth, in the belief that an understanding of the processes involved might provide unique opportunities to study one of nature's immunosuppressive mechanisms. On the other hand, it is conceivable that identifying ways to restore T cell responsiveness in patients with severe active RA could also be beneficial in terms of restoring T cell-dependent immunoregulatory networks, host defense and antitumour immunity. Our experimental approach to exploring how TNF uncoupled TCR signaling pathways was primarily influenced by the observation that TNF attenuated T cell calcium responses following peptide stimulation,[29] and by studies indicating that TNF increased the threshold of T cell activation.[30]

Using a mouse T cell hybridoma model, we made several novel observations. First, chronic TNF lowered the expression of cell surface TCR/CD3 complexes. By immunoprecipitation analysis, we discovered that levels of the TCRζ chain, but not the CD3γ, δ, or ε chains, associated with the TCR/CD3 complex were selectively downregulated.[30] This was confirmed by Western blotting of whole cell lysates. In contrast, expression of the protein tyrosine kinases Lck, Fyn, and ZAP-70 in TNF-treated T cells was spared. The reductions in cell surface TCR expression could be explained by selective loss of TCRζ, since TCRζ is required for TCR assembly and transport to the cell surface, and its synthesis is rate-limiting, being approximately 10% of that of the other invariant CD3 chains.[36] These findings were of particular interest given the data from several laboratories demonstrating that TCRζ chain expression was downregulated in RA synovial T cells compared with peripheral blood T cells from the same patients.[37,38]

As well as being important for TCR/CD3 assembly and expression, TCRζ chain functions as a signal amplification module since it carries three immunoreceptor tyrosine-based activation motifs (ITAM) in the cytoplasmic domain, as opposed to the single ITAMs of CD3ε, γ, and δ chains.[39,40] Indeed, it has been possible to correlate the strength of the TCR signal with degrees of phosphorylation of TCRζ ITAMs.[41] By comparing the proximal TCR signalling events in control and TNF-treated T cells, we were able to show that TCRζ phosphorylation was attenuated in TNF-treated T cells, even though the ratio of partially to fully phosphorylated TCRζ phospho-species was not different from that of the control.[30] This suggested that phosphorylation of TCRζ ITAMs per se by src family kinases was spared, and that the reduction in phospho-TCRζ, as well as reductions in phospho-CD3ε, was secondary to the loss of TCRζ protein expression. This was confirmed when we subsequently demonstrated normal Lck kinase activity in TNF-treated T cells. It is likely that reductions in ZAP-70 and p36 LAT phosphorylation arise through the same mechanisms, since we have been unable to detect loss of protein expression or kinase activity that could not be accounted for by the defects in TCRζ expression. The precise mechanisms for the downregulation of TCRζ expression by TNF are not known. However, TCRζ mRNA is reduced in T cells treated with higher concentrations of TNF.[30] Furthermore, TNF may also impair signaling indirectly through the upregulation of reactive oxygen species, since culture of TNF-treated T cells with the glutathione precursor N-acetylcysteine reverses some, but not all, of the signalling defects we had documented in TNF-treated T cells.[30]

These results raise several intriguing issues. First, they indicate that TNF may attenuate T cell responses by uncoupling proximal TCR signalling pathways; whether downstream pathways are attenuated independently of TCRζ downregulation is under investigation. Second, given the results of similar experiments in RA synovial T cells, they also suggest that TNF may contribute to the loss of TCRζ expression observed in RA synovial T cells.[37,38] This can now easily be tested in patients treated with anti-TNF, in whom it would be predicted that levels of TCRζ are restored. Third, the notion that the inflammatory environment may regulate TCRζ expression predicts that its

expression might be abnormal in other chronic inflammatory diseases. Indeed, several lines of evidence indicate that this is indeed the case. For example, TCRζ protein and gene expression has been found to be abnormal in T cells from patients with SLE (reviewed by Tsokos and co-workers[42,43]; see additional references therein).

While studies of peripheral blood T cells from lupus patients and synovial T cells from RA patients reveal consistent downregulation of TCRζ expression,[37,38,44–46] the mechanisms for these defects appear to be distinct. In RA, the published data suggest that loss of TCRζ expression and phosphorylation in T cells from inflamed joints may be related to the redox potential, since the culture of synovial T cells with N-acetylcysteine substantially reverses synovial T cell hyporesponsiveness.[47] While this finding would be consistent with a posttranslational effect, perhaps at the level of TCR/CD3 assembly and the dimerisation of TCRζ through disulphide bonds, additional transcriptional and post-transcriptional/translational mechanisms have not been explored. In SLE, the data are more complex. TCRζ expression and phosphorylation are downregulated in lupus T cells,[44–46] and yet, unlike RA synovial T cells, there is evidence for the hyperphosphorylation of signalling molecules as well as increased inositol triphosphate levels and calcium mobilisation, albeit transient.[44,48] The mechanisms for this paradox are not clear. On the other hand, studies of TCRζ gene expression suggest that TCRζ defects in lupus patients may be inherited, at least in part. For example, TCRζ mRNA transcripts are absent in some patients, and through alternative splicing, deletions of exon 7 (among others) have been reported.[45,49] More recently, heterogeneous mutations and polymorphisms have been identified throughout the TCRζ sequence; some have been identified in cDNA but not in the corresponding genomic sequence,[50] invoking complex processes such as genomic editing. Finally, polymorphisms within the promoter and 3′ untranslated region suggest that the TCRζ gene may be regulated at both transcriptional and posttranscriptional levels.[51] It should be pointed out that these mutations do not appear to be disease-specific; nor do they correlate with disease activity. Together, the data suggest that the regulation of TCRζ expression in chronic inflammatory disease is a complex multifactorial process, in all probability dictated by both genetic and environmental factors. Given the critical role for the TCR/CD3 complex in transducing proximal TCR signalling pathways, defective TCRζ regulation in inflammation could promote autoimmunity through aberrations in central and peripheral tolerance, as suggested from experiments in TCRζ-deficient mice.[52]

The data have further implications for understanding T cell differentiation in chronic inflammation, since they suggest that signals emanating from TCR expressed on T cells in the joint may contribute considerably less than those from TCR expressed on naïve T cells exposed to specific MHC/peptide complexes for the first time. At the same time, signals derived from cytokine receptors such as the TNF-R would be correspondingly increased. According to this model, T cell effector responses are driven by chronic cytokine activation rather than by antigenic stimulation, at least in terminally differentiated, senescent T cells. From this, one might predict that cytokine receptor signalling pathways are chronically activated in synovial T cells.

The published data in this field are sparse, and since many signalling pathways are shared between the TCR and cytokine receptors, it has been difficult to establish precisely which receptors are stimulating the relevant pathways. Nonetheless, recent studies suggest that there is some evidence for activation of the mitogen-activated protein kinases (MAPK) extracellular signal-related kinase (Erk), c-Jun N-terminal kinase (Jnk), and p38 MAPK (p38),[53] as well as NF-κB in synovial T cells.[54] In the context of uncoupled TCR signalling pathways, it seems reasonable to explore the possibility that chronic activation of these pathways is maintained not by antigen, but by proinflammatory cytokines such as IL-1 and TNF. Indeed, recent unpublished data from our laboratory suggests that MAPK and NF-κB pathways are clearly induced in activated T cells following stimulation by TNF. More importantly, the profile of activation pathways induced by short-term TNF stimulation is quite distinct in T cells chronically exposed to low concentrations of TNF, when compared with the pathways activated by TNF in T cells that are TNF "naïve." Preliminary data provide evidence for selective desensitisation of some downstream pathways but not others (P. Vagenas and A.P. Cope, unpublished data). Consistent with these findings are recent results demonstrating the suppression of collagen-induced arthritis with agents that selectively inhibit NF-κB in T cells.[55] These data lend further support to the idea that not only is there a change in the balance of signals delivered by TCR and cytokine receptors in chronically activated T cells, but also that there are additional qualitative differences in the cytokine receptor signals imposed by the duration of cytokine exposure.

What are the implications of these results for patients? A detailed analysis of patients treated with anti-TNF has revealed that, in addition to potent anti-inflammatory effects, T and B cell responses are restored. This is manifest by increases in peripheral lymphocyte counts, facilitated by a reduction in the trafficking of leucocytes to the synovial joint, enhanced peripheral blood proliferative and cytokine responses at the single-cell level, and in approximately 8% of patients the development of antinuclear antibodies (reviewed by Feldmann and Maini[56]). Recovery from T cell hyporesponsiveness could explain to some extent the surprisingly low incidence of serious infections which might have been predicted based on the established role for TNF in innate immunity. Even so, great care must be taken when screening for intracellular pathogens in these patients, both before and during therapy.

The schematic diagram in Fig. 1 summarises how T cell differentiation might progress in a host who is either nonsusceptible or susceptible to disease. According to this model, a productive T cell effector response of appropriate magnitude and duration is generated in the nonsusceptible host, but is subsequently terminated by regulatory mechanisms, which are themselves dependent on intact TCR signalling pathways. The model proposes that in the susceptible host these mechanisms fail, leading to the persistence

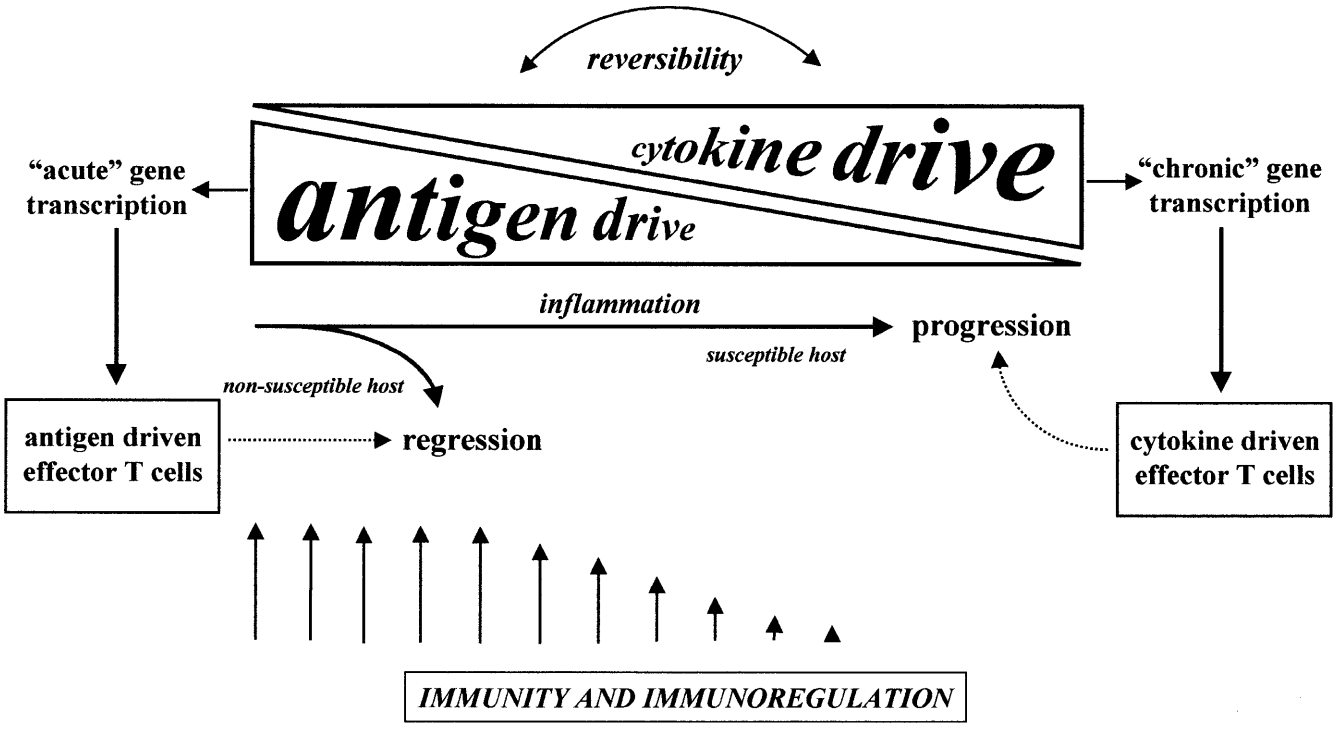

Fig. 1. A model of T cell differentiation in chronic inflammation. The schematic diagram depicts how the relative contributions of TCR and cytokine receptor signalling may influence the programme of gene transcription in a disease-susceptible or nonsusceptible host. The possibility that some degree of reversibility may exist provides opportunities for therapeutic intervention

of T cell activity and the propagation of effector T cells sustained more by cytokine activation than by antigen stimulation. Central to this model is the idea that, while suppression of antigen reactivity could represent an adaptive response of the host to attenuate the inflammatory process, loss of TCR signalling would impair thymic function, peripheral tolerance mechanisms including activation-induced cell death, and the generation in the thymus and the function in the periphery of immunoregulatory T cells. Indeed, there is evidence to suggest that following anti-TNF treatment there is enhanced T cell apoptosis in the synovial joints of RA patients, and in the gut lining of Crohn's patients.[57] It is possible that the restoration of immunity and immunoregulatory function resets the adaptive immune system. This process of resetting might go some way to explaining some of the disease-modifying effects of TNF blockade.[56] It could also explain the long-lasting effects of a single infusion of anti-TNF, which may be sustained for weeks or even months after the therapeutic agent has cleared from the body.

Finally, replicative senescence and attenuated proliferative and cytokine responses have suggested to some workers that synovial T cells are just "burnt out and stressed out." Is it possible that T cells with such a phenotype could still exert effector functions? The pioneering work of Dayer and co-workers,[58,59] and studies from other laboratories including our own, suggest that this may be the case. Thus, chronically activated T cells could drive the inflammatory process through cell-to-cell contact-dependent mechanisms, independently of antigen stimulation. Through cell-surface receptor–ligand interactions, T cells may activate macrophages to produce inflammatory cytokines, B cells to produce autoantibodies, and fibroblasts to produce inflammatory cytokines and matrix metalloproteinases. The prediction from this is that the chronic TNF-induced programme of gene transcription will include genes encoding cell-surface antigens that are capable of generating inflammatory signals through direct cell-to-cell contact. Integrins and CD69 are just some of the candidates which have been tested and found to promote such interactions.[60] The development of proteomic and genomic techniques to screen for candidate molecules should greatly facilitate such studies. It is our belief that these surface antigens, together with the intracellular signalling pathways that promote these responses, could provide the new generation of therapeutic targets for the future.

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