Supplement Review

Studies of T-cell activation in chronic inflammation

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Chapter summary

The strong association between specific alleles encoded within the MHC class II region and the development of rheumatoid arthritis (RA) has provided the best evidence to date that $CD4^+$ T cells play a role in the pathogenesis of this chronic inflammatory disease. However, the unusual phenotype of synovial T cells, including their profound proliferative hyporesponsiveness to TCR ligation, has challenged the notion that T-cell effector responses are driven by cognate cartilage antigens in inflamed synovial joints. The hierarchy of T-cell dysfunction from peripheral blood to inflamed joint suggests that these defects are acquired through prolonged exposure to proinflammatory cytokines such as tumour necrosis factor (TNF)-α. Indeed, there are now compelling data to suggest that chronic cytokine activation may contribute substantially to the phenotype and effector function of synovial T cells. Studies reveal that chronic exposure of T cells to TNF uncouples TCR signal transduction pathways by impairing the assembly and stability of the TCR/CD3 complex at the cell surface. Despite this membrane-proximal effect, TNF selectively uncouples downstream signalling pathways, as is shown by the dramatic suppression of calcium signalling responses, while Ras/ERK activation is spared. On the basis of these data, it is proposed that T-cell survival and effector responses are driven by antigen-independent, cytokine-dependent mechanisms, and that therapeutic strategies that seek to restore T-cell homeostasis rather than further depress T-cell function should be explored in the future.

Keywords: inflammation, rheumatoid arthritis, signal transduction, T cells, TNF

Introduction and historical background

Evolving concepts of disease mechanisms for rheumatoid arthritis (RA) have provided a paradigm for understanding the pathogenesis of autoimmune disease. This paradigm proposes that genetic and environmental factors shape a complex series of molecular and cellular interactions leading to a chronic inflammatory response. $CD4^+$ T lymphocytes have featured prominently because the genetic elements most strongly associated with RA susceptibility or severity are encoded within the MHC class II region (discussed in this issue in the chapters by H McDevitt, and G Sønderstrup). Precisely how effector T cells initiate and promote the inflammatory process in RA, however, remains far from clear. Much effort has focussed on establishing the molecular nature of antigenic reactivity, in the belief that the established chronic phase of the disease is antigen-driven. Animal models of inflammatory arthritis would certainly lend support to this view. However, the results of detailed phenotypic and functional analyses of chronically activated T cells derived from inflamed joints are difficult to reconcile with traditional models of cartilage-antigen-driven inflammatory disease in patients with RA. This chapter aims to explore this theme in more depth, beginning with an outline of the molecular events that

A glossary of specialist terms used in this chapter appears at the end of the text section.

dictate the differentiation of T helper (Th) cells at the outset of adaptive immune responses in regional lymph nodes. Much of the remainder of the discussion focuses on the different ways in which, in the longer term, the chronic inflammatory process influences maturation, differentiation, and function of effector T cells at sites of inflammation. I conclude by speculating about how our understanding of T-cell activation in chronic inflammation may influence future therapy, and discuss this in the context of the prevailing view that in a susceptible host, chronic inflammatory disease occurs through a failure of regulatory T cells to downregulate the inflammatory process.

Acquisition of transcriptional competence during differentiation of T helper cells

There is now good evidence that there exists a coordinated programme of molecular events initiated at the outset of T-cell differentiation that leads to the generation of $CD4^+$ Th effector cells [1]. This process of differentiation is characterised by a distinctive pattern of cytokine production and is important because its outcome dictates the host response to foreign pathogens such as *Listeria monocytogenes* infection or to parasitic infestation [2]. For cytokine genes, at least three stages are thought to be required for the acquisition of transcriptional competence in T cells: an initiation phase, a commitment phase, and a phase of acute gene transcription (Fig. 1). The existence of these stages has been deduced largely from experiments in which monospecific T cells are stimulated *in vitro* in bulk cultures from naïve precursors [3,4]. During the initiation phase, naïve T cells are engaged through their T-cell receptors (TCRs) by MHC/peptide complexes expressed on the surface of dendritic cells. Only those T cells that form a functional immunological synapse are likely to differentiate [5]. At this point, intracellular signalling pathways emanating from stable clusters of TCR/CD3 complexes are integrated with those from cytokine receptors following engagement by cytokines. For example, IL-12 and IL-18 are important for differentiation of Th1 cells but also play a key role in innate immunity. In polarised Th cells, some cytokines activate families of transcription factors called 'signal transducers and activators of transcription', or STATs, such as STAT4 for IL-12 (Th1), or STAT6 for IL-4 (Th2) [6]. The initial engagement phase may last from hours to days, and in general will result in the production of IL-2 and entry of the cell into the cell cycle. At this stage, cells are incapable of producing Th1 (IFN-γ) or Th2 (IL-4) cytokines, despite optimal stimulation by antigenic peptide and cytokines. Moreover, surprisingly few T cells progress beyond this early stage of maturation.

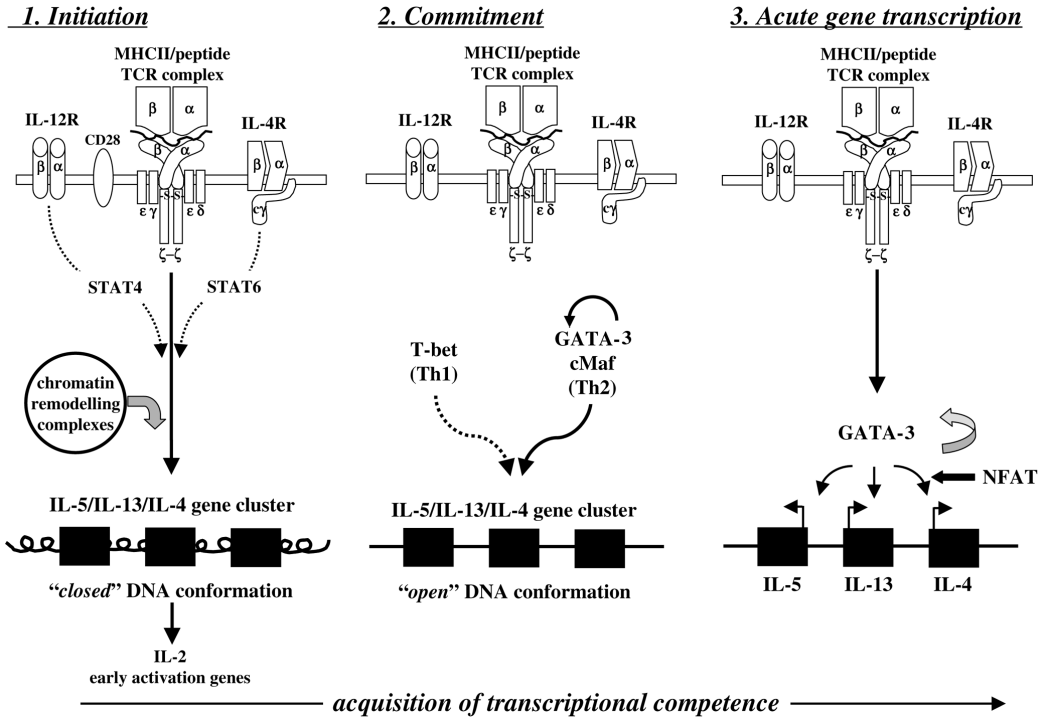
The commitment phase is characterised at the molecular level by the induction and recruitment of Th-subset-specific transcription factors. Those considered to be lineage-specific include GATA-3 and c-Maf for Th2 cells [7,8], and T-bet and ERM for Th1 cells [9,10]. Once these factors are expressed, differentiation is stabilised and maintained even in the absence of further TCR stimulation (see Fig. 1, middle panel). The third phase, that of acute gene transcription, is determined by secondary contact with antigen and necessitates the recruitment of nuclear factor of activated T cells (NFAT) together with subset-specific transcription factors to the transcriptosome complex (see Fig. 1, right panel). This process is thought to be monoallelic and stochastic, probably because it depends upon chromatin accessibility [1,6]. Thus, specific loci become transcriptionally active through a series of changes to chromatin structure, including chromatin decondensation and remodelling, and recruitment of complexes to the nuclear matrix [11]. Within 48 hours of stimulation, new clusters of DNase-hypersensitive sites can be detected, as demonstrated for the IL-4 gene [12], possibly through the coordinated action of STATS and other transcription factors such as the binding of p300 and of calcium-binding proteinC/EBP (CCAAT/enhancer binding protein) to DNA elements [1,6]. These sites are markers of stable, differentiated T cells. Coincident with these changes in the nucleus are overall increases in histone acetylation, histone phosphorylation, and DNA demethylation [13,14], which occur during the S phase of the cell cycle [15].

Full commitment to a specific lineage is established gradually and in most cases takes place in regional lymph nodes. According to this model, Th cells can be thought of as being in 'antigen mode', since the transcriptional programme required for effector function is absolutely determined by antigen and TCR signalling pathways. It follows from this that the expression and stability of the TCR on the T-cell surface, its avidity for MHC/peptide complexes, the signal strength, and the integrity of protein tyrosine kinases and signalling adaptor molecules would be essential in determining both qualitative and quantitative characteristics of the immune response [16].

T-cell differentiation in chronic inflammation – clues from the rheumatoid joint

While these molecular events go some way towards explaining why some $CD4^+$ T cells differentiate into Th1 cell subsets and produce IFN-γ while others are destined to become Th2 cells producing IL-4, IL-5, and IL-13, much less is known about the events that regulate T-cell effector responses in the context of chronic immunoinflammatory responses. Part of the problem lies in accessibility to T cells at sites of inflammation available for study, the heterogeneity of T-cell subsets recruited to these sites, the unpredictable immunomodulatory effects of drug therapy, and the relapsing and remitting nature of the disease process itself. Notwithstanding these issues, it has been possible to draw some tentative conclusions based on the phenotype of T cells isolated from inflamed tissue such as the RA synovial joint.

Figure 1



Acquisition of transcriptional competence during differentiation of T helper cells. Th cells become productive effectors of immunoinflammatory responses following a complex series of molecular events dependent upon membrane-proximal TCRs and cytokine receptor signals. Chromatin remodelling is an essential step in the process leading to a switch from the 'closed' to 'open' DNA conformation. This in turn permits accessibility of Th-subset-specific transcription factors and accessory factors to the promoter elements of the Th2 gene cluster, as illustrated here. Ultimately, NFAT is recruited to the transcriptosome, after which cytokine gene transcription proceeds. c-Maf, transcription factor specific for Th2 cells; ERM, transcription factor specific for Th1 cells; GATA-3, transcription factor specific for Th2 cells; NFAT, nuclear factor of activated T cells; STAT, signal transducer and activator of transcription; T-bet, transcription factor specific for Th1 cells; TCR, T-cell receptor; Th, T helper (cell).

What do the available data tell us about T-cell activation and differentiation in established chronic inflammation? The evidence for chronic immune activation is unambiguous (Table 1). This is best illustrated by histological analyses of sections of synovial tissue in which, in subsets of patients with more severe RA, there exist perivascular follicular lymphoid-like structures resembling germinal centres of lymphoid organs [17,18]. Lymphoid aggregates in synovial tissue are rich in T cells, B cells expressing MHC class II, and dendritic cells, and their precise cellular organisation is thought to depend on the local expression of cytokines and chemokines [19]. *Ex vivo,* flow cytometric analysis reveals a memory phenotype for synovial tissue and fluid T cells expressing CD45RO but low levels of CD45RB [20,21], suggestive of past or persistent antigenic stimulation. Their cell surface carries other markers of activation, such as CD69, CD44, and HLA-DR, as well as the chemokine receptors CCR4, CCR5, CXCR3, and CX_3CR1, whose selective expression may facilitate homing to synovial joints [20,22]. Synovial T cells persist in the joint, possibly through an environment that favours cell survival. The expression of stromal cell derived survival factors such as IFN-α may contribute [23]. The demonstration of both significant and premature telomere shortening would also suggest that these cells undergo progressive self-replication *in situ* [24], and so by the time the inflammatory process is established, subsets of synovial T cells may already be approaching the stage of terminal differentiation or senescence.

While there is a general consensus that in inflamed synovial joints there is enrichment of Th1 T cells [25], the demonstration of significant populations of polarised Th1 subsets has been difficult, even with the advent of intracellular staining techniques [26,27]. Indeed, expression of cytokines cannot be detected easily without stimulation, and even after stimulation with anti-CD3 and anti-CD28, the frequency of cytokine-expressing cells may be very low, necessitating pharmacological stimulants (e.g. phorbol ester and ionophore) to demonstrate the presence of cytokine-producing T cells. Nonetheless, the finding of a paucity of Th2-cytokine-expressing T cells by many laboratories has been more consistent [25,28], and recently there are data to suggest that naïve RA peripheral

Table 1

Characteristics of chronically activated T lymphocytes in the synovium of patients with rheumatoid arthritis

1	T lymphocytes are found in follicular lymphoid aggregates
2	The cell-surface phenotype is suggestive of chronic immune activation, e.g. expression of CD45RO, CD69, and subsets of chemokine receptors
3	T cells are terminally differentiated, with significant telomere loss
4	Synovial T cells are hyporesponsive to TCR ligation
5	Synovial T cells exist in an environment favouring cell survival
6	There is an imbalance of pro- and anti-inflammatory cytokines, with a predominance of macrophage products in inflamed joints
7	There is a bias towards the development of T helper (Th)1 cells

blood T cells may be refractory to Th2 differentiation [29]. According to these data, there exists *in vivo* an imbalance between proinflammatory cytokines and anti-inflammatory cytokines whereupon a deficiency of anti-inflammatory cytokines might be expected to favour the failure of immunoregulatory mechanisms [30]. Taken together, these experimental observations go some way towards supporting the idea that in inflamed synovial joints, the T cell is in a state of chronic immune activation, if not to say a state of chronic stress or exhaustion, and that the default regulatory mechanisms operating in the nonsusceptible host are absent or insufficient.

Synovial T cells are hyporesponsive to TCR engagement

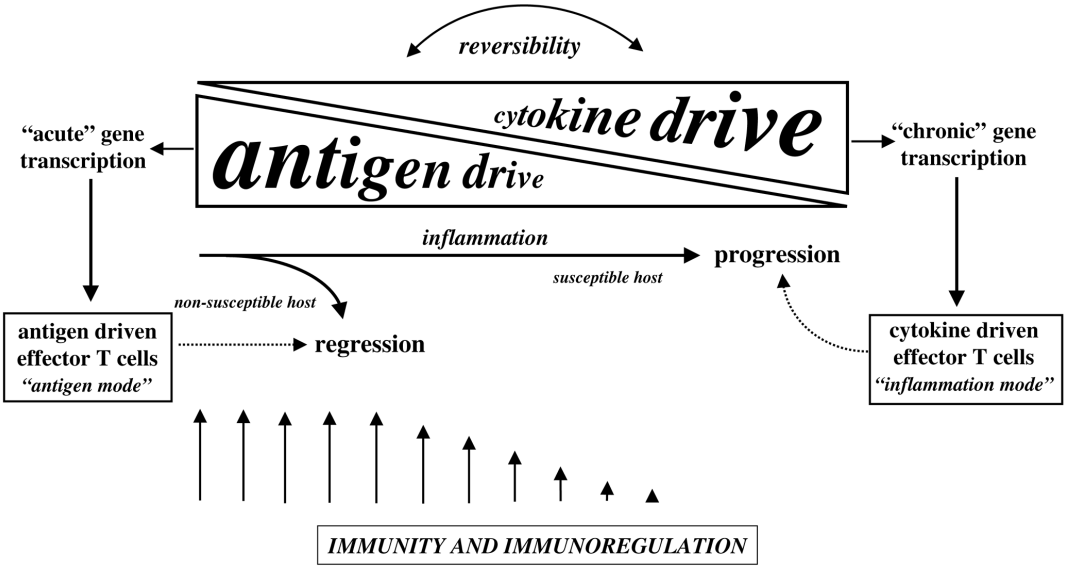
The low levels of constitutive cytokine expression in synovial T cells is puzzling, given their activated phenotype. More puzzling is the finding that they proliferate very poorly *in vitro* in response to either mitogen, recall antigens, or CD3 ligation with specific agonistic monoclonal antibodies (Table 1, and [31–33]). Indeed, suppression of proliferative and cytokine responses has led some workers in the field to conclude that terminally differentiated T cells may not contribute to the established inflammatory response [34]. However, this does not seem compatible with the histopathological features of inflamed joints outlined above.

There are several possible reasons for the unusual phenotype of synovial T cells, which may have to do with both spatial and temporal parameters. Firstly, the anatomy and cellular environment of inflamed synovial tissue are different from the architecture and cellular constituents of lymph nodes, with hypoxia and extremes of extracellular pH imposing significant pathophysiological effects (discussed in this issue in chapters by P Taylor and E Paleolog). Secondly, extensive analysis of the inflammatory environment demonstrates that the cytokine milieu in RA synovial tissue is different from that expressed in a lymph node during primary interactions between dendritic cells and precursors of Th cells, with a strong predominance of macrophage products [30]. Accordingly, the expression of cytokine receptors and the acquisition of chronic cytokine responsiveness is likely to be a distinguishing feature of chronically activated T cells in inflamed joints. Historically, it has been assumed that T-cell activation in the rheumatoid joint would be driven by peptide fragments of cartilage antigens presented by disease-associated MHC-class-II molecules [35,36]. However, this model now has to take into account the fact that synovial T cells are hyporesponsive to TCR engagement. These findings raise the possibility that during the evolution of immune and inflammatory responses, the balance of stimulation shifts from 'antigen mode', in which T cells are engaged through the TCR/CD3 complex during the early phases of Th differentiation, to 'inflammation mode', in which T-cell activation and effector responses are driven by proinflammatory cytokines (Fig. 2).

A model of T-cell activation in chronic inflammation

Almost a decade ago, we began to think about ways to explore how the chronic inflammatory process might influence T-cell autoreactivity and effector responses, in the belief that this might contribute to an understanding of the immunopathogenic processes involved in the chronic phase of RA. Our approach was influenced to a great extent by a series of observations arising from a larger programme of work in the laboratory and by others, which sought to document in depth the broad range of cytokines expressed in rheumatoid joints. The observations of particular importance included the findings that tumour necrosis factor (TNF)-α bioactivity persists in synovial joint cell cultures and *in vivo* [37–40]; that both high-affinity p55 and p75 tumour-necrosis-factor receptors (TNFRs) are upregulated on synovial joint T cells [41]; that TNFRs are expressed in lymphoid aggregates and colocalise with ligand [42]; and that expression of the naturally occurring TNF inhibitors, the soluble TNFRs, is also increased in synovial fluid but is insufficient to completely neutralise bioactive TNF *in vivo* [43]. The implication of these findings was that synovial mononuclear-cell infiltrates, including T cells, are chronically exposed to TNF *in vivo*. We

Figure 2



A model for the role of CD4+ T cells in the pathogenesis of chronic inflammation. Antigen drive predominates during the early phase of inflammatory responses ('antigen mode'). In a nonsusceptible host, the immune response resolves through mechanisms such as activation-induced cell death and/or the production of immunoregulatory cytokines. In the susceptible host, additional T cells are recruited to sites of inflammation through bystander activation, or by stimulation with self antigens released from inflamed tissues. As the inflammatory process progresses, chronic cytokine production induces profound nondeletional T-cell hyporesponsiveness. Hyporesponsive T cells function as effector cells and sustain the chronic inflammatory process through predominantly antigen independent mechanisms ('inflammation mode'). It is proposed that by reversing T-cell hyporesponsiveness, antigen-dependent responses that serve to regulate the inflammatory process (e.g. through expression of immunoregulatory cytokines) are restored.

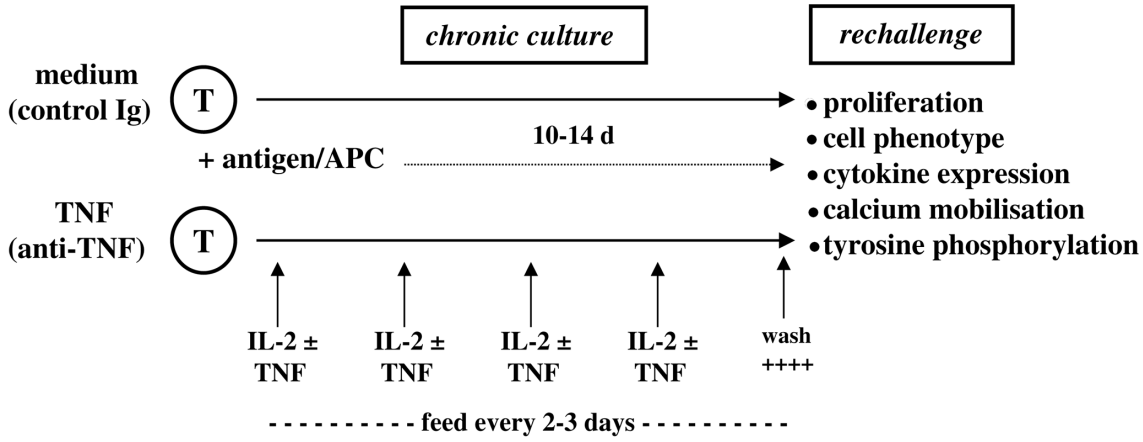
therefore predicted that the environment generated in chronically inflamed joints must be very different from that provided by an acute inflammatory or infectious episode (Table 1), and that chronic exposure to inflammatory cytokines might have effects distinct from those induced after short-term exposure.

We set out to mimic chronic exposure to cytokines by culturing antigen-activated T cells in the presence of TNF. This *in vitro* model was very similar to that used in many laboratories to explore the effects of cytokines such as IFN-γ, IL-4, and IL-12 on T-cell differentiation [3,4], with the exception that recombinant TNF was added repeatedly to T-cell cultures to mimic better the sustained TNF signalling we believed existed in inflamed joints (Fig. 3) [44]. The principal finding that chronic, as opposed to acute, exposure to TNF suppressed T-cell activation was unambiguous, and could not have been predicted from published data at that time, which suggested that TNF was costimulatory and a growth factor for T cells [45]. These results have been confirmed in other laboratories [46–48] and have been supported further through extensive analyses of both human and murine T-cell lines and clones *in vitro,* as well as from experiments *in vivo* undertaken in T-cell receptor transgenic mice treated with recombinant TNF, anti-TNF, or after intercrossing to human-TNF-globin transgenic mice [49,50]. The findings are summarised in Table 2. Two lines of evidence convinced us that these unexpected findings were potentially important and worthy of further investigation. The first was the observation that peripheral blood T-cell responses from patients with RA were dramatically and rapidly restored after treatment with anti-TNF (infliximab, Remicade™) [44] and that these immunological parameters closely followed clinical improvement [51]. The second line of evidence came from a series of studies undertaken in TCR transgenic mice. These experiments revealed that repeated injections of otherwise healthy transgenic mice with anti-TNF enhanced T-cell responses to cognate peptide antigen, and implied that physiological concentrations of TNF had immunomodulatory properties *in vivo* [49].

The immunomodulatory effects of TNF in the initiation and resolution of autoimmunity

Until the late 1980s, it was assumed that TNF was both proinflammatory and co-stimulatory [30]. How could these initial observations be reconciled with the immunosuppressive effects outlined above? An extensive series of studies in mice deficient for TNF or TNFR have confirmed that TNF has potent immunomodulatory effects *in vivo*, capable of regulating T-cell autoreactivity and autoimmunity when

Figure 3



Model for studying the effects of TNF on T-cell differentiation and maturation. T cells are stimulated with cognate antigen in the presence of irradiated antigen-presenting cells for periods of up to 14 days in the presence or absence of recombinant TNF. Cytokines are added to cultures every 2 or 3 days. At the end of the culture period, T cells are washed extensively and then rechallenged with specific antigen or anti-CD3 mAb in the absence of TNF. APC, antigen-presenting cell; T, T cell; TNF, tumour necrosis factor-α.

Table 2

Characteristics of CD4+ T cells chronically exposed to tumour necrosis factor

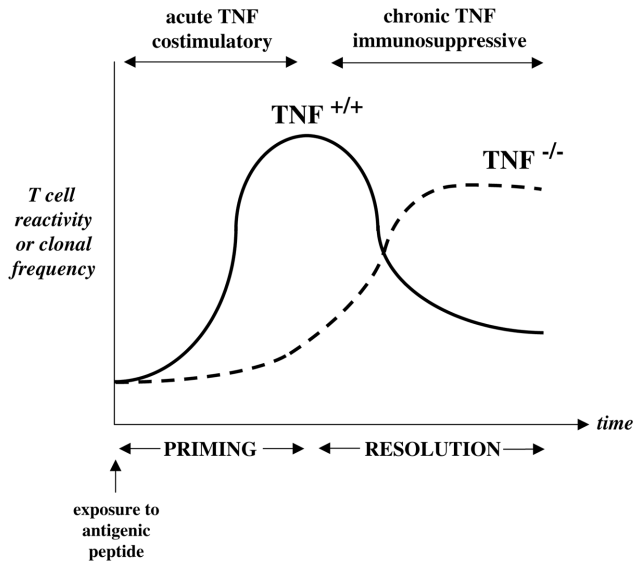
1	Upregulation of activation antigens such as CD69
2	Induction of nondeletional, proliferative hyporesponsiveness
3	Suppression of cytokine production
4	Uncoupling of TCR signal transduction pathways
5	Repression of CD28 gene transcription

studied in autoimmune-susceptible strains of mice [50]. Indeed, the suppressive effects of chronic TNF were entirely consistent with the studies of Jacob and McDevitt [52], as well as Gordon and colleagues [53], who first demonstrated the disease-protecting effects of chronic TNF therapy in the NZB/W F_1 lupus-prone mouse. Similar effects were reported subsequently in murine models of type I diabetes [54,55]. Using a mouse model of multiple sclerosis, Kollias and colleagues demonstrated that while acute TNF exposure is important for T-cell priming to cognate antigen, chronic TNF is required for resolution of T-cell reactivity to myelin antigens [56]. They found that the initiation of T-cell reactivity to myelin basic protein or myelin oligodendrocyte glycoprotein in TNF-deficient mice of the H-2^b strain, which is normally resistant to experimental autoimmune encephalomyelitis, was dramatically impaired, consistent with the idea that TNF was an absolute pre-requisite for T-cell priming by antigen. However, by following the T-cell responses to self-antigens over time, it became apparent that while antigen reactivity peaked and then declined to concentrations no longer detectable in wild-type mice, responses gradually increased and became sustained in TNF-deficient littermates many weeks after immunisation. This sustained and uncontrolled autoreactivity to myelin oligodendrocyte glycoprotein correlated closely with the development of a chronic demyelinating disease in an otherwise disease-resistant strain [56]. These data provide compelling evidence to suggest that while short-term TNF is important for antigen priming, sustained TNF expression is necessary for resolution of T-cell responses (Fig. 4).

How does chronic TNF attenuate T-cell activation?

The potent immunodulatory effects of prolonged TNF exposure *in vitro* and *in vivo* in both mouse and man has prompted us to explore in more depth the molecular and biochemical basis for these findings, in the belief that an understanding of the processes involved might unravel one of nature's immunosuppressive mechanisms. Therefore, we began to study TNF effects on T-cell hybridomas, since these cells could be propagated in the absence of accessory cells. Using this model, we found that the suppression of IL-2 production (to 10% of that in control T cells) was the most profound that we had observed to date. Our first series of experiments revealed that chronic TNF stimulation increased the threshold for T-cell activation through the TCR, such that more peptide/MHC complexes were required for longer periods of time for TNF-treated T cells to commit to IL-2 production [57]. Closer scrutiny of TNF-treated T cells revealed both dose- and time-dependent reductions in expression of the TCR/CD3 complex at the cell surface, as determined by flow cytometry or by cell surface immunoprecipitation experiments. In contrast, levels of expression of CD3ε in

Figure 4



The immunomodulatory effects of TNF during the evolution of the immune response. After TCR ligation, TNF is costimulatory and required for antigen priming. As the immune response proceeds over time, TNF is required to suppress subsequent clonal expansion ($TNF^{+/+}$; unbroken line). In TNF-deficient animals ($TNF^{-/-}$; dotted line), immune responses are delayed, but, once established, they fail to resolve, leading to persistent antigen reactivity. TNF, tumour necrosis factor-α.

whole-cell lysates from the same cells were unimpaired [57]. An understanding of the process of TCR/CD3 complex assembly provided the first clues as to the most likely mechanism for this unexpected observation.

TNF impairs assembly and stability of the TCR/CD3 complex at the cell surface

Current concepts of TCR/CD3 complex assembly have been based largely on detailed molecular analyses in T-cell hybridomas using metabolic labelling and pulse-chase experiments. They describe a process where, for full function, the polymorphic TCRαβ chains associate with the invariant chains (CD3 γ, δ, and ε and TCRζ) consisting of noncovalently linked γε and δε heterodimers and disulphide-linked ζ–ζ homodimers, which transmit signals inside the cell (Fig. 5). Association of TCRζ dimers with newly synthesized hexameric complexes (αβγεδε) results in the transport and subsequent expression of the complete TCR/CD3 complex ($\alpha\beta\gamma\varepsilon\delta\varepsilon\zeta_2$) at the cell surface. Studies in T-cell hybridomas have revealed that TCRζ is synthesized at ~10% of the rate of other components [58], and therefore the amount of TCRζ available in a given T cell is thought to regulate TCR/CD3 expression at the cell surface.

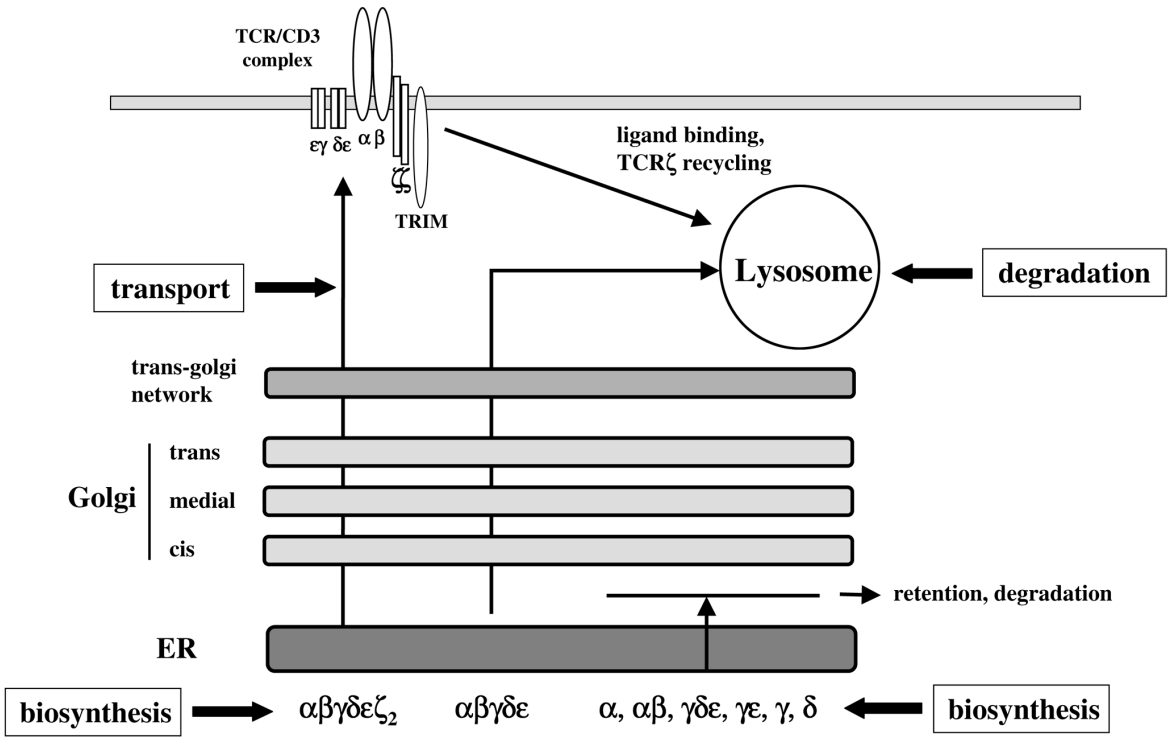
Our results predicted that expression of TCRζ may be one target of chronic TNF stimulation. Indeed, following closely the kinetics of IL-2 downregulation, western blotting analysis of whole-cell lysates revealed that chronic stimulation with TNF suppressed the expression of TCRζ in a dose- and time-dependent fashion, while concentrations of the protein tyrosine kinases ZAP-70, p56Lck, and p59Fyn were not altered [57]. Furthermore, immunoprecipitation of CD3ε-containing complexes revealed normal concentrations of CD3ε, γ and δ, indicating that TNF had selective effects on TCRζ expression. Given that TCRζ might be rate limiting for TCR/CD3 assembly, the data were consistent with a model in which TNF appeared to disrupt the assembly of TCR/CD3 complexes through its effects on TCRζ expression. Profound reduction in concentrations of cell-surface biotinylated TCRζ in TNF-treated T cells strongly supported this notion [57].

A second unexpected experimental observation provided further evidence that persistent TNF signalling in T cells could perturb TCR/CD3 expression at the cell surface. Immunoblotting analysis of unstimulated and TNF-stimulated T cells revealed that the expression of the novel transmembrane adaptor protein TRIM (T-cell-receptor-interacting molecule) was markedly downregulated by TNF treatment [59; Isomäki and Cope, unpublished data]. Closer examination revealed that TRIM expression was reduced by TNF before changes in TCRζ expression could be detected, and that reconstitution of both TRIM and TCRζ expression was required to fully restore TCR responsiveness in TNF-treated cells. The implications of these findings have only recently become apparent, through studies of TRIM expression in human peripheral blood and Jurkat T cells. In collaborative studies with Dr Burkhart Schraven, it was found that the half-life of TCR/CD3 complexes in stable Jurkat clones overexpressing TRIM is increased [60]. This in turn leads to increased cell-surface expression of TCR and enhanced signalling responses as determined by intracellular calcium mobilisation. We can conclude from these experiments that sustained TNF signals in T cells impair TCR/CD3 assembly not only through its effects on TCRζ expression, but also by reducing the half-life of assembled complexes at the cell surface by downregulating the expression of TRIM (see Fig. 5). The kinetics of these changes, as well as the precise interactions between TCRζ and TRIM, are now being studied. Nevertheless, the findings provided a molecular basis for the profound hyporesponsiveness of T cells after TNF stimulation and predicted that downstream TCR signalling pathways might be significantly attenuated as a consequence of these structural constraints.

TNF attenuates membrane-proximal TCR signalling pathways

One of the earliest events detected after TCR ligation is the phosphorylation of tandemly arranged tyrosine residues within immunoreceptor tyrosine-based activation motifs (ITAMs) of TCRζ chain and CD3γ, δ, and ε chains

Figure 5



Assembly and degradation of the TCR/CD3 complex. TCRζ and TRIM are required for assembly and stability of the TCR/CD3 complex at the cell surface (see text for further details). ER, endoplasmic reticulum; TCR, T-cell receptor; TRIM, T-cell-receptor-interacting molecule.

by Src family kinases, notably Lck and Fyn [61]. In contrast to CD3 chains, which contain just one ITAM, TCRζ carries three, providing the TCR/CD3 complex with a signal sensor and amplification module [62,63]. In addition, TCRζ plays a role in proofreading extracellular signals, since differences in the quality, intensity, and duration of the antigenic stimulus are translated into specific patterns of TCRζ phosphorylation [64]. Once phosphorylated, TCRζ ITAMs function as docking sites for protein tyrosine kinases of the Syk family, such as ZAP-70 [65]. The phosphorylation of several adaptor proteins by ZAP-70 and Src kinases then serves as a link between membrane-proximal phosphorylation events and the activation of downstream signalling pathways leading to IL-2 production, T-cell proliferation, and effector responses [61].

Given that TCRζ functions as a signal-amplification module as well as a key component of TCR/CD3 complex assembly, we reasoned that reduced expression of TCRζ homodimers by TNF might impair membrane-proximal tyrosine phosphorylation events. A comprehensive analysis of signalling pathways in control and TNF-treated T cells has shown that concentrations of phospho-TCRζ are reduced in TNF-treated T cells after TCR ligation [57]. Furthermore, in spite of normal Lck kinase activity, the recruitment of ZAP-70 to phospho-TCRζ through its SH2 domains and its subsequent phosphorylation were also impaired. The transmembrane adaptor protein linker for activation of T cells (LAT) is an *in vivo* substrate for ZAP-70 kinase, and plays a key role in linking membrane-proximal events with both calcium and Ras/MAPK (mitogen-activated protein kinase) pathways [66,67]. LAT phosphorylation was substantially reduced in TNF-treated cells, and, as predicted, intracellular calcium mobilisation was also dramatically attenuated [57]. The precise mechanisms for the down-regulation of TCRζ expression by TNF are not clear. However, TCRζ mRNA is reduced in T cells treated with higher concentrations of TNF (2.5 ng/ml). Furthermore, TNF may also reduce TCRζ concentrations indirectly through the generation 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, possibly by restoring TCRζ expression [57]. Regardless of the mechanisms, the data were consistent with a model in which proximal signalling was impaired as a direct result of the effects of TNF on TCRζ expression and phosphorylation and suggested a novel mechanism whereby the inflammatory process might suppress T-cell reactivity in RA synovial joints.

Selective uncoupling of downstream signalling by TNF

Since the transmembrane adaptor protein LAT functions as a pivotal bifurcation point for downstream Ras/ERK (extracellular signal-regulated kinase) and calcium signalling pathways [67], the reductions in concentrations of phosphorylated LAT would predict that these downstream pathways should be attenuated in TNF-treated T cells. Several lines of preliminary evidence suggest that this prediction may be too simplistic. We have been struck by the extent to which TCR-induced calcium responses are attenuated in TNF-treated T cells [49,57]. However, very recent experiments have documented additional defects in calcium signalling that arise through mechanisms independent of the effects of TNF on membrane-proximal phosphorylation events and TCR/CD3 expression. For example, while TNF depletes to only a modest extent the thapsigargin-depletable intracellular calcium pool, TNF attenuates to a much greater extent the influx of calcium through store-operated I_{CRAC} (calcium-release-activated calcium current) channels ([68], and Fig. 6). The activation of I_{CRAC} calcium channels and influx of extracellular calcium contributes significantly to the amplitude, duration, and kinetics of the total calcium signal, which in turn is known to profoundly influence gene expression in T cells [69]. We believe that this may provide an additional mechanism through which TNF attenuates gene transcription through calcium/NFAT-dependent pathways, and may go some way to explain the profound proliferative and cytokine hyporesponsiveness following TCR ligation that we have observed after chronic TNF exposure.

We next undertook a systematic analysis of the Ras/ERK pathway in control and TNF-treated T cells, expecting to document similar degrees of attenuation. The results were unexpected. While concentrations of TCR-induced GTP-Ras are only modestly reduced in TNF-treated T cells, phosphorylation of Raf-1, activation of ERK1/2, induction of c-Fos, and TCR-induced expression of CD69 are unambiguously preserved. These results would predict that AP (activator protein)-1 transactivation is likely to be spared in TNF-treated T cells. If this is found to be the case, the data define a novel biochemical basis for acquired suppression of T-cell activation based upon selective attenuation of the calcium but not of the Ras/ERK pathways (Fig. 7). At the biochemical level, this result is of particular interest given the observations that anergic T cells have reciprocal defects, namely, reductions in TCR-induced Ras/ERK activation, while calcium responses are spared [70,71].

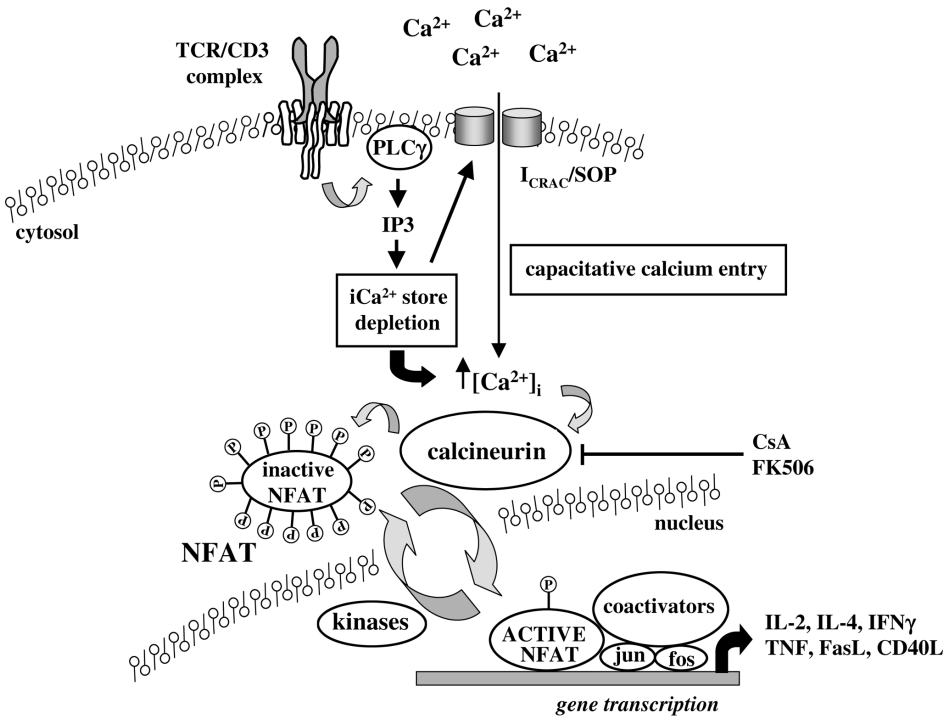
Aberrant signal transduction pathways in synovial T cells in rheumatoid arthritis

The effects of chronic TNF described above have provided an experimental framework for exploring the role of inflammatory cytokines in regulating the phenotype of synovial T cells from inflamed joints of patients with RA. Many of the features of T cells chronically exposed to TNF resemble RA synovial T cells (see Tables 1 and 2). Most notable among these are the nondeletional, reversible T-cell hyporesponsiveness [31–33], the upregulation of cell-surface antigens [20], and the repression of CD28 gene expression [72]. These similarities suggest that TNF may play a role in driving this phenotype, and raise the intriguing possibility that there may also be similarities in terms of the aberrations of intracellular signalling pathways that might account for this phenotype.

The pioneering studies of Verweij and colleagues in this field have perhaps most comprehensively and systematically documented proximal TCR signalling in synovial-fluid T cells [73]. The most relevant to this discussion is the downregulation of TCRζ chain expression in synovial-fluid T cells in comparison with peripheral blood, as determined by flow cytometry, immunoblotting, or immunohistochemistry [73,74]. Expression and phosphorylation of p36LAT and its recruitment to the plasma membrane is also reduced in synovial-fluid T cells [75], and both this phenomenon and the loss of TCRζ expression can be reversed at least partially by restoring concentrations of glutathione by culturing T cells *ex vivo* with *N*-acetylcysteine [73,75]. This is an important finding, since it indicates that TCR signalling pathways are sensitive to reactive oxygen species and redox potential. More recent data indicate that the generation of such oxygen species is regulated by Ras, which is itself expressed in a constitutively active GTP bound form in synovial T cells, and that increased concentrations of these oxygen species may influence the tertiary structure and conformation of LAT at the plasma membrane [76].

The similarities between synovial T cells and T cells generated *in vitro* after repeated TNF stimulation extend further to calcium responses, including influx through I_{CRAC} channels, which are also attenuated in peripheral blood and joint T cells from patients [77,78], as well as in Jurkat T cells treated with TNF [79]. Using a completely different approach, Isaacs and colleagues have compared the characteristics of anergic $CD4^+$ T cells and RA synovial T cells at the mRNA level by differential display RT-PCR [80]. One striking transcriptional event common to both sets of T cells was the downregulation of calmodulin, a gene whose product plays an important role in coupling calcium responses to downstream pathways. Indeed, transcription of calmodulin in RA synovial T cells was less than 1% that in synovial samples from patients with reactive arthritis, who served as the controls in these studies. Expression in synovial T cells was lower than that observed in paired peripheral blood T cells. Interestingly, calmodulin transcripts increased 5- to 10-fold after TNF blockade *in vivo* in all six patients studied. Thus, the findings of impaired calcium responses on the one hand, and constitutively active Ras on the other, provide compelling evidence of

Figure 6



The calcium/calcineurin/NFAT signalling pathway in T cells. After TCR ligation and PLCγ1 activation, newly synthesized IP3 binds to tetrameric IP3 receptor complexes inducing the release of intracellular calcium stores from the sarco-endoplasmic reticulum. Store depletion leads directly to the opening of I_{CRAC} or store-operated channels (SOC) in the plasma membrane through mechanisms that are unclear. This leads ultimately to activation of the serine phosphatase calcineurin, dephosphorylation of NFAT, and translocation of this transcription factor to the nucleus. For many genes, NFAT binds cooperatively to AP-1 complexes for optimal gene transcription. AP, activator protein; CsA, cyclosporin A; iCa^{2+}, intracellular calcium; I_{CRAC}, calcium-release-activated calcium current; IP, inositol phosphate; P, phosphate group; PLCγ, phospholipase Cγ; TCR, T-cell receptor.

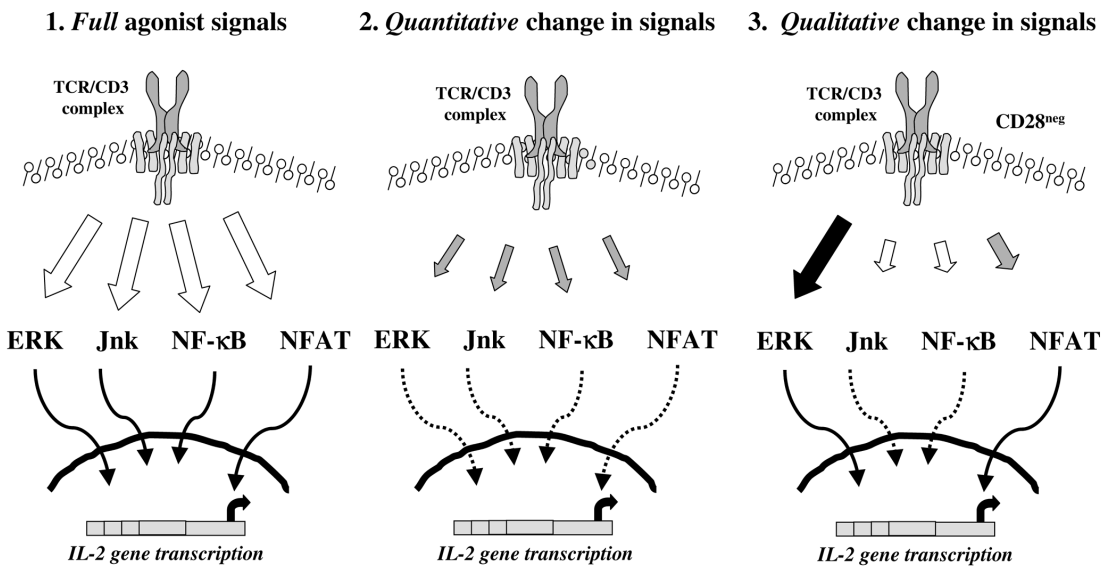
selective uncoupling of TCR signalling in chronically active T cells *in vivo*, similar to that observed in TNF-treated T cells (see Fig. 7). Moreover, direct comparisons of peripheral blood and joint T cells have established hierarchical attenuation of signalling pathways from peripheral blood to the joint, with the most profound defects being observed in the joint [73–75], suggesting that these signalling anomalies are not inherited but are likely to be acquired through chronic exposure to the environment in inflamed joints over prolonged periods of time.

Are there any clues suggesting selective activation of downstream signalling pathways leading to specific transcriptional events at the nuclear level? Data up to now are scarce, but there are isolated reports of constitutive NF-κB activation in RA synovial T cells [81]. The finding of constitutive activation of NF-κB in synovial T cells is of particular interest given the recent studies demonstrating the beneficial therapeutic effects of a T-cell selective NF-κB inhibitory compound (SP100030) in collagen-induced arthritis [82]. The same group have documented attenuation of adjuvant arthritis in rats using dominant negative IKKβ (inhibitor of NF-κB kinase β) delivered by adenoviral vector [83], although when this approach is used, inhibition of NF-κB would not be confined to T cells. In addition, a recent analysis of constitutive MAPK activation in synovial tissue suggests that there may be preferential activation of ERK in lymphoid aggregates in perivascular tissue [84]. It is perhaps premature to draw any firm conclusions about which transcription factors are active in synovial T cells, other than to state that there is evidence for constitutive activation of pathways *in vivo* that exert a potential for promoting the inflammatory process, and perhaps cell survival.

Implications for the pathogenesis of chronic inflammatory disease

The effects of TNF on downstream TCR signalling pathways outlined above, together with the studies of calcium signalling in synovial T cells from patients with RA, predict that transactivation of NFAT should be dramatically reduced in the synovial joint. If this indeed is the case, then it provides a molecular framework for exploring further how the inflammatory process might influence

Figure 7



TCR signal transduction pathways. Engagement and stabilisation of TCR/CD3 complexes leads to a membrane-proximal cascade of tyrosine phosphorylation events that ultimately lead to the activation of kinases and transcription factors directly involved in gene transcription. Ligation of TCR and costimulatory receptors leads to activation of multiple pathways, including ERK, JNK, NF-κB, and NFAT (left panel). Impaired assembly and stability of the TCR/CD3 complex would be expected to attenuate all downstream pathways (middle panel). However, chronic TNF stimulation leads to selective uncoupling of TCR signalling, such that TCR-induced calcium/NFAT responses are impaired, while Ras/ERK activation is spared (right panel). The effects of proinflammatory cytokines on the activation of these specific pathways are not included here. ERK, extracellular signal-regulated kinase; Jnk, c-Jun N-terminal kinase; NF, nuclear factor; NFAT, nuclear factor of activated T cells; TCR, T-cell receptor.

immunity and inflammation *in vivo*. For example, NFAT is required for the transcription of many genes involved in the initiation of the immune response, cell growth and differentiation, the induction of immunoregulatory cytokines, host defence, and resolution of the immune response through activation-induced cell death [85]. Accordingly, defects in this pathway would lead not only to depressed immunity and the failure to generate productive Th effector responses, but also to the failure of tolerance by impaired TCR-induced expression of FasL (Fas ligand), attenuation of activation-induced cell death, and the failure to mount significant immunoregulatory responses.

Our own studies of the effects of chronic TNF signalling emphasise the potential for cytokine-dependent, antigen-independent effector mechanisms, driven perhaps through chronic stimulation of the NF-κB pathway. Sustained NF-κB activation would promote trafficking to sites of inflammation, as well as enhance the survival of cells in the inflamed joint, thereby promoting effector responses dependent on cell-to-cell interactions [86,87]. Preliminary phenotyping and genotyping analyses in our laboratory suggest that a number of potential candidates may be upregulated on the cell surface of TNF-treated T cells as a direct consequence of chronic NF-κB activation. These include RANK (receptor activator of nuclear factor κB) ligand, whose upregulation enhances osteoclastogenesis and bone resorption; β integrins, which promote T-cell trafficking to inflamed joints; and CD69, a surface antigen that has been shown to promote signalling between macrophages and T cells and inflammatory cytokine production [87]. This switch from 'antigen mode' to 'inflammation mode' and the generation of antigen-independent effector responses in T cells (see Fig. 2) suggests that conventional therapeutic approaches for modulating T-cell reactivity may need to be revised.

Future prospects for therapy

It has long been recognised that cellular immunity and, in particular, T-cell activation are restored after treatment with remission-inducing therapy, regardless of the disease-modifying agent. We now know that anti-TNF treatment is no exception [44]. The question of whether inflammatory disease remits as a consequence of the recovery of immune competence or in spite of it has never been addressed in depth. On the basis of the available data, we can only conclude that recovery of T-cell reactivity is compatible with attenuation of the disease process and does not seem to exacerbate the inflammatory process. The possibility that normalisation of function of a subset of specific T cells with anti-inflammatory activity occurs is consistent with this conclusion.

Results from several laboratories, including our own, suggest that therapeutic strategies aimed at restoring T-cell homeostasis should be given serious consideration, and should in addition take into account the effects of the inflammatory process on thymic function [50,80,88,89]. This therapeutic approach is in line with the thesis proposing that susceptibility to autoimmunity arises not through clonal expansion of autoaggressive effector T cells as a primary event, but more through the failure of the adaptive immune system to regulate an inflammatory response [90].

How could recovery of T-cell regulatory activity be achieved in man? A major challenge in the short term will be to define more precisely a phenotype for regulatory T-cell subsets so that their frequency can be studied in peripheral blood and at sites of inflammation. It would be of particular interest to establish whether their TCR signalling responses and regulatory function correlate inversely with disease activity. Such studies might include analyses of the new generation of 'suppressor' T cells such as IL-10-producing, Tr1-like $CD4^+$ cells, $CD4^+CD25^+$ regulatory T cells, or IL-16-producing $CD8^+$ T cells [91–94]. If, in the longer term, antigenic specificity can be established, T-cell responsiveness could be restored towards 'normal' (but *not* beyond) by combining peptide therapy with anti-TNF. Precise knowledge of the specific TCR signalling defects could facilitate the monitoring of such therapy, so that any potential for rebound hyper-reactivity of bystander T cells and its deleterious consequences, including systemic autoimmune disease, could be substantially reduced [95,96]. Anti-TNF in combination with nondepleting anti-CD4 or anti-CD3 mAb might have similar beneficial therapeutic effects.

There exists an alternative to the hypothesis that the acquisition of T-cell hyporesponsiveness promotes the inflammatory process. For example, defective T-cell reactivity at sites of inflammation may turn out to be an essential adaptive response for suppressing autoreactivity, as suggested by studies in mice (50,88). In this event, it would be important to understand how the inflammatory process uncouples signalling pathways, since this might facilitate the development of novel immunosuppressive agents. Rather than target pathways that are already suppressed, such as the calcium/calcineurin pathway, these strategies might attenuate those that are dominant and that drive the inflammatory process. According to this model, the Ras/ERK and NF-κB signalling pathways would be good candidates.

Concluding remarks

The molecular events that shape the early phase of Th cell differentiation in regional lymph nodes are quite likely to be distinct from those imposed by the environment of an inflamed joint. It follows from this that the design of strategies for manipulating T-cell effector responses should be governed by the chronicity of the immune response. Until now, therapies that target T cells have been based largely upon the potency of agents in acute immunoinflammatory responses in the laboratory. With a growing knowledge base of the characteristics and phenotype of chronically activated T cells, we can look forward to a new generation of therapeutics targeting selective intracellular pathways involved directly in promoting the chronic inflammatory process. Whether such strategies will necessitate targeted immunoablation or restoration of T-cell homeostasis remains to be seen. However, when considering the available options, it should be borne in mind that protection against foreign pathogens is the primary function of the immune system and that this immunity is provided to the host at the expense of a huge propensity for cross-reactivity to self tissue antigens. My own bias, which is derived in part from the working model illustrated in Fig. 2, would be to focus on methods of reconstituting the immune system of patients with the regulatory networks that keep this cross-reactivity in check and the vast majority of individuals in good health.

Glossary of terms

c-Maf = a transcription factor specific for Th2 cells; ERM = a transcription factor specific for Th1 cells; I_{CRAC} = calcium-release-activated calcium current; ITAM = immunoreceptor tyrosine-based activation motifs; GATA-3 = a transcription factor specific for Th2 cells; T-bet = a transcription factor specific for Th1 cells.

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