Review

Exploring the reciprocal relationship between immunity and inflammation in chronic inflammatory arthritis

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Experimental models seeking to explore how susceptible individuals develop rheumatoid arthritis (RA) propose that genetic and environmental factors shape a complex series of molecular and cellular interactions leading to a chronic inflammatory response. T lymphocytes and MHC class II genes have featured prominently in these models. More recent studies have suggested that perpetuation of inflammation in a disease-susceptible host might occur through failure to down-regulate the inflammatory process. One prediction from this model is that effective mechanisms of immunoregulation might be most easily investigated in non-susceptible individuals. However, this has been difficult to study in man. Based on the observation that extended MHC haplotypes are strongly associated with RA in different ethnic groups, I have explored the function of human MHC-encoded genes in transgenic mice using two different experimental approaches. First, by comparing the molecular interactions between disease-associated or non-associated HLA-DR4 molecules and CD4+ T lymphocytes, it has been possible to gain insight into how immune responses in non-susceptible individuals might differ from T-cell responses observed in a susceptible host. This has been achieved using transgenic mice expressing RA disease-associated and non-associated human HLA class II molecules. Secondly, the effects of prolonged exposure of T cells to the proinflammatory cytokine tumour necrosis factor α (TNF) have been studied in vitro and in vivo, focusing on T-cell receptor (TCR) signalling and effector responses. In studies of HLA class II transgenic mice, the major differences between disease-associated and non-associated alleles in terms of T-cell responses occur at the level of presentation of antigenic peptides, and the sustained expression of inflammatory cytokines such as TNF. Chronic exposure of T cells to inflammatory cytokines such as TNF induces a phenotype which resembles RA synovial T cells, including the induction of non-deleitional and reversible hyporesponsiveness to TCR ligation and uncoupling of proximal TCR signal transduction pathways. The experimental findings are consistent with a model in which HLA class II-driven inflammatory cytokine expression uncouples TCR signalling pathways in the susceptible host in such a way as to profoundly suppress proliferative and immunoregulatory cytokine responses, while at the same time promoting cell survival and effector responses.

Key words: Autoimmunity, Rheumatoid arthritis, T lymphocytes, HLA-DR4, Tumour necrosis factor, T-cell activation, T-cell hyporesponsiveness.
Protection against foreign pathogens is the primary function of the immune system. While the innate immune system provides the first line of defence, adaptive immunity and the acquisition of memory responses to foreign antigens is achieved through the generation of an extensive repertoire of lymphocyte antigen receptors. Because this repertoire is generated during thymic maturation and maintained in the periphery through recognition of complexes of self peptide and major histocompatibility complex (MHC) molecules, all peripheral T cells have the potential for autoreactivity. Whilst thymic maturation generates T lymphocytes expressing low-avidity T-cell receptors (TCR) at low precursor frequencies, this recognition mechanism cannot discriminate between self and foreign antigens. Therefore, protective immunity is provided to the host at the expense of a huge propensity for cross-reactivity to self tissue antigens.

How is it that autoimmunity is very common while autoimmune disease is quite rare?

Over the last few years, susceptibility to autoimmunity has been explored not so much in terms of a predisposition to generating autoaggressive effector cells, but more in terms of failure to regulate an autoimmune response [reviewed in 2]. If one considers current concepts of cognate immunity, a defect in immunoregulation could be acquired at multiple points during both early and later phases of an autoimmune response. For CD4+ T lymphocytes, the initial ligand–receptor interaction between MHC–peptide and the TCR is crucial, since this determines qualitative and quantitative aspects of the signals transduced through the TCR [3]. Seen through the eyes of an autoreactive T cell, the evolution of autoimmune disease could be envisaged as a series of checkpoints (Fig. 1). These checkpoints provide a theoretical framework for understanding not only how disease might progress in a susceptible host, but also for exploring how the evolution of autoimmune responses might differ qualitatively in the non-susceptible host.

Exploring the function of disease-associated HLA-DRB1 genes in rheumatoid arthritis

How do we begin to study in man the complex molecular interactions that contribute to the pathogenesis of RA? For many laboratories, the choice of experimental strategy

![Diagram](https://academic.oup.com/rheumatology/article-abstract/42/6/716/1788211/Downloaded_on?date=27_April_2019)
has been guided by genetic studies which have focused on defining gene polymorphisms which most clearly discriminate between patients with RA and the non-susceptible host. Some of the strongest associations found in RA patients from different ethnic groups to date are with MHC class II genes [5, 6]. This association was first described by Stastny in the 1970s [7], but a significant advance in our understanding of these associations was reported more than a decade later, when it was shown that susceptibility to RA across different ethnic populations correlated closely with the expression of a specific consensus amino acid sequence (the ‘shared epitope’) within the HLA-DRβ1 chain [8]. This sequence was subsequently shown by several groups of investigators to be encoded by HLA-DRB1 alleles, including HLA-DR4 (*0401, *0404, *0405 and 0408), but also HLA-DR1 (*0101), DR6 (*1401) and DR10 (*1001) alleles [9–11]. Significantly, some of the key polymorphisms within non-associated DRB1*04 alleles mapped to the same region of the DRβ chain α helix (Table 1). For example, DRβ1*0402 carries negatively charged residues at β70 and 71, as opposed to the positively charged residues found at β70 in disease-associated DRB1 alleles, while DRβ1*0403 (non-associated), which differs from *0404 (associated) by one amino acid, carries a negatively charged residue at codon 74.

These associations, together with the finding of follicular aggregates of lymphocytes and antigen-presenting cells (APC) in the synovial tissue of patients with active RA, have provided perhaps the strongest evidence that the immune response contributes to disease chronicity. By studying these associations in patient cohorts that are clinically as well as genetically heterogeneous, it has been possible to identify genotypes that cosegregate with specific clinical features. For example, in population studies, different HLA-DRB1 alleles appear to influence the severity of disease, DRB1*0401 being found in patients with severe, seropositive, erosive RA (often with extra-articular features, such as vasculitis and Felty’s syndrome, in *0401-homozygous or *0401/*0404 compound homozygous individuals), while DRB1*0101 and *1001 are observed at a higher frequency in patients with less severe, seronegative, non-erosive disease [12]. Inheriting two copies of alleles expressing the consensus sequence greatly increases disease penetrance, time of onset and severity. These and other studies have suggested that genetic associations may contribute more to severity than to disease susceptibility [reviewed in 4]. Closer inspection suggests further that, rather than a hierarchy of phenotypes of a single disease, these genetic associations, which are by no means uniform, are reflected clinically as distinct disease entities. These distinct entities may be manifest at the level of synovial histomorphology [13], somewhat analogously to the histological features that define different forms of B-cell lymphoma.

**A functional basis for the associations between HLA-DR and RA**

MHC class II molecules function by selecting and presenting immunogenic peptide fragments of protein antigens to CD4+ T cells. They also play a role in the selection of the TCR repertoire in the thymus. Because CD4+ T cells recognize linear stretches of about 9–20 amino acids derived from self or foreign protein antigens bound in the peptide-binding groove of polymorphic MHC class II molecules, it has been suggested that differences in the way that HLA-DR or -DQ molecules present selected peptides to T cells could be an important mechanism for susceptibility to autoimmune diseases such as RA. According to this model, RA might represent the sequelae of pathological T-cell responses initiated and maintained by antigens presented by disease-associated HLA class II molecules. On the basis of early observations, two principal models were proposed to account for the association between RA and the consensus DRβ chain sequence. Both were based on the assumption that the shared epitope is the critical genetic element linked directly to disease. The first model proposed that the shared epitope determines specific peptide binding, and that ‘pathogenic’ peptides bind only to disease-associated HLA class II molecules [14, 15]. This model predicted that a gradient of affinities of disease-inducing peptide for MHC class II molecules might account for the differences in susceptibility and/or severity conferred by different HLA-DR molecules. The second model proposed that the shared epitope influences TCR recognition by binding and selecting autoreactive T cells during thymic maturation, and expanding these populations in the peripheral compartment [16, 17].

Although these models are not mutually exclusive, critical testing of the models in the context of RA has been the subject of intense research in many laboratories.

<table>
<thead>
<tr>
<th>HLA-DRB1 allele</th>
<th>Pocket 1</th>
<th>Pocket 4</th>
<th>Pocket 6</th>
<th>Pocket 7</th>
<th>Pocket 9</th>
<th>RA</th>
</tr>
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<tbody>
<tr>
<td>DRB1*0101</td>
<td>G</td>
<td>F</td>
<td>Q</td>
<td>R</td>
<td>A</td>
<td>L</td>
</tr>
<tr>
<td>DRB1*0401</td>
<td>H</td>
<td>G</td>
<td>Q</td>
<td>K</td>
<td>A</td>
<td>V</td>
</tr>
<tr>
<td>DRB1*0402</td>
<td>⌀</td>
<td>⌀</td>
<td>⌀</td>
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<tr>
<td>DRB1*0403</td>
<td>V</td>
<td>H</td>
<td>Q</td>
<td>R</td>
<td>E</td>
<td>V</td>
</tr>
<tr>
<td>DRB1*0404</td>
<td>V</td>
<td>H</td>
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<td>A</td>
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<td>H</td>
<td>Q</td>
<td>R</td>
<td>A</td>
<td>V</td>
</tr>
</tbody>
</table>

Circles denote amino acid residues in the DRβ1*0402 molecule that differ in DRβ1*0401. +++, strong association with RA; –, weak or no association with RA.
Progress in this area has been hampered largely by the fact that, until recently, it has not been possible to compare the function of disease-associated and non-associated HLA-DR4 molecules in isolation under experimental conditions where the remainder of the genome is fixed.

**Studying HLA-DR4-restricted T-cell responses in transgenic mice**

Conceptually, an experimental approach involving the generation of transgenic mice expressing human MHC class II molecules has proved attractive. Such an approach has been developed by a number of laboratories with the intention of studying the function of disease-associated HLA class II molecules in vivo in a way that had not been previously possible in human subjects [18–20]. Comprehensive reviews published recently have detailed the experimental approaches, highlighting the constraints of such models and discussing how these limitations have been resolved [21, 22]. This approach in mice has turned out to be enormously useful for probing in vivo the function of disease-associated and non-associated human MHC class II molecules, and their potential for contributing to the development of inflammatory arthritis [23, 24].

**Generating a HLA-DR4-restricted TCR repertoire in transgenic mice**

Using DR4 transgenic mice that my colleagues and I had generated at Stanford University, it was first necessary to study in detail a CD4\(^+\) T-cell response specific for cognate antigen that was restricted to the HLA-DRB1*04 allele encoding HLA-DR\(\beta\)1*0401, and to characterize the immunogenic epitopes presented by these HLA-DR4 molecules in vivo. This allele was chosen because of its strong associations with RA in Caucasians [11]. To this end, it was necessary to generate a repertoire of CD4\(^+\) T cells whose TCR had been shaped by HLA-DR4 during thymic maturation. This was only achieved after a detailed analysis of the peripheral CD4\(^+\) T-cell compartment in transgenic mice expressing different genotypes [25]. For example, while I-A\(^\beta\)0 mouse MHC class II-deficient mice have <0.5% mature CD4\(^+\) T cells in the peripheral blood [26], introduction of a single copy of the HLA-DR4 transgene onto this mouse class II-deficient background increased this level to around 4%. After crossing to human CD4-transgenic mice, the peripheral CD4 compartment increased further to 13%, while homozygous HLA-DR4/human CD4-transgenic I-A\(^\beta\)0/0 mice had peripheral mouse CD4\(^+\) T-cell numbers approaching those observed in mice expressing endogenous mouse MHC class II I-A molecules (~25–30%) [22, 25]. These observations, combined with the fact that HLA-DR4 had been shown to alter the repertoire of selected V\(\beta\) TCR in I-A-expressing mice [18], demonstrated that human HLA-DR4 can shape, both quantitatively and qualitatively, the TCR repertoire in mice. It also provided a molecular framework for exploring in depth the specificity of mature CD4\(^+\) T cells that had previously undergone maturation and selection through cognate interactions with self-peptide/HLA-DR4 complexes in the thymus of transgenic mice.

**What do HLA-DR4-restricted T cells really see?**

HLA-DR4-restricted T-cell responses were defined using human cartilage (HC) gp-39 as a model antigen [27]. Although HCgp-39 is expressed in many tissues, it is produced in abundance by chondrocytes, and protein is present in synovial membrane as well as synovial fluid and serum [28–30]. The finding that mRNA transcripts are not detectable in healthy cartilage explants but can be induced in tissue from arthritic joints, as well as by proinflammatory cytokines in vitro, made this an attractive candidate antigen for study [28, 29]. Through the generation of immortalized T-cell hybridomas, the specificities of literally thousands of T-cell responses were evaluated. From more than 250 HCgp-39-specific responses, nine immunogenic epitopes were identified, with frequencies of responses of T cells to specific peptides ranging from <1% to as high as 35% of the T-cell response, indicating that this was a sensitive as well as a specific method for studying HLA-DR4-restricted T-cell responses in vivo [27]. Epitopes 100–115, 262–277 and 322–337 contributed around 80% of the total peptide specificity (Table 2), but, surprisingly, not one of these peptides carried the predicted charged residue at position 4. More than 95% of all antigen-specific responses were restricted to HLA-DR4, the remainder being restricted to the DR\(\alpha\)1-E\(\beta\) cross-species heterodimer, which is also expressed in these mice, albeit at very low levels. All responses could be blocked with anti-DR monoclonal antibodies, and each epitope identified was subsequently found to be processed and presented efficiently by human DR\(\alpha\)1*0401-expressing APC. In a pilot study designed to validate the relevance of HCgp-39 epitopes defined in mice, all immunogenic epitopes of HCgp-39 were capable of eliciting peripheral blood

### Table 2. Characteristics of immunodeficient epitopes of HCgp-39 defined in HLA-DR4-transgenic mice

<table>
<thead>
<tr>
<th>Peptide residue</th>
<th>Frequency of HLA-DR4 restricted hybrids</th>
<th>Core motif relative position</th>
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<tbody>
<tr>
<td></td>
<td>*0401 (n = 250)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>*0402 (n = 141)</td>
<td></td>
</tr>
<tr>
<td>100–114 (106–114)</td>
<td>20</td>
<td>F S K I A S N T Q</td>
</tr>
<tr>
<td>262–277 (265–273)</td>
<td>34</td>
<td>F T L A S E T G</td>
</tr>
<tr>
<td>322–337 (328–336)</td>
<td>27</td>
<td>Y D D Q E S V K S</td>
</tr>
<tr>
<td>22–37 (25–33)</td>
<td>0</td>
<td>V C Y Y T S W S Q</td>
</tr>
<tr>
<td>298–313 (302–310)</td>
<td>0</td>
<td>L R G A T V H R T</td>
</tr>
</tbody>
</table>

Adapted from reference 27 and reviewed in more detail in 22.
T-cell proliferative responses in at least some patients with RA carrying HLA-DRB1*04 alleles encoding the shared epitope consensus sequence [27]. This approach made it possible to define precisely, for HLA-DR4-restricted T cells, the immunodominant epitopes of a cartilage antigen that elicited T-cell responses in patients with RA, and as such defined at the molecular level the first checkpoint, defined as cartilage antigen peptide-specific recognition by TCR (checkpoint 1, Fig. 1). According to these data, TCR recognition appeared to be highly specific for a large number of clones, because strict dependence on specific peptide for reactivity was demonstrated and TCR triggering did not occur in the absence of specific peptide. However, more detailed analyses indicated that seemingly monospecific T cells were also cross-reactive.

**Immunization with HCgp-39 promotes reactivity to self-peptide–self-MHC complexes**

While screening for positive responses to HCgp-39, a subset of hybrids (~15–20%) were found to be highly reactive to transgenic but not non-transgenic splenic APC in the absence of exogenous antigen [31]. Similar responses were observed when DRα/β1*0401-expressing Epstein-Barr virus-transformed B cells were used as APC. These responses were directed to complexes of self peptide and HLA-DR4, since they could be inhibited by anti-DR monoclonal antibodies.

Detailed analysis of self-reactive T cells revealed great flexibility of TCR recognition at multiple levels [32]. Activation of a subset of clones was further enhanced by the addition of HCgp-39 or a panel of peptides derived from HCgp-39. These intramolecular peptide mimics, while carrying predicted HLA-DR-binding motifs, carried sequences which were quite distinct from each other at the amino acid level. These clones also responded to APC expressing closely related HLA-DRB1 alleles that encoded the shared epitope consensus sequence β67–74, with or without exogenous antigen. Responses to DRα/β1*0401 and to *0405 APC were particularly vigorous, while responses to DRα/β1*0402 and *0403 were never observed (Fig. 2).

These data provided direct evidence for diversity and flexibility of TCR recognition early in the evolution of the immune response, and demonstrated that cross-reactivity to self-peptide–self-MHC complexes following immunization with cognate antigen is frequent. For many responses, MHC-biased recognition by TCR predominated. For these T cells, one might predict charge complementarity between MHC shared epitope sequences and TCR complementarity-determining regions (Fig. 3B), as proposed recently [33], rather than between MHC and cognate peptide (Fig. 3A). This cross-reactivity would also provide a mechanism through which critical precursor frequencies of autoreactive T cells could be achieved, and, because of MHC-directed recognition, could evolve by propagation through interactions largely independent of specific antigen. According to this model, the peptide is permissive for TCR recognition (checkpoint 2, Fig. 1). While the fact that the repertoire of TCR is shaped on self-peptide–self-MHC complexes during thymic maturation provides the most plausible explanation for this, the model provides further evidence that cross-reactivity of peripheral T cells is universal [1]. In the light of these observations, it should be possible to demonstrate broad self-reactivity for all TCR, including populations of peptide-specific T cells that are apparently monospecific, seemingly lacking the broad flexibility of other T-cell subsets. Because immune responses to foreign antigens are generated from the same pool of potentially self-reactive TCR, it follows from this that immunization of HLA-DR4-transgenic mice with proteins derived from foreign pathogens would activate and expand populations of autoreactive T cells.

**A) Responses to different peptide ligands**

**B) Responses to different APC alone**

![Graph A) Responses to different peptide ligands](image1)

**Fig. 2.** Flexibility of TCR recognition by HCgp-39-specific T cells. (A) T cells were stimulated with APC plus HCgp-39 peptides or native antigen. Note high background counts (>100 000 IL-2 fluorescence units) in the absence of antigen. (B) T cells were stimulated with a panel of DRB1-expressing APC in the absence of exogenous antigen. Data are shown for clones 4A10 and 1A2, both specific for peptide 262–277. Results are expressed as arbitrary IL-2 fluorescence units.
Self-reactivity following immune responses to foreign pathogens

In collaboration with the Kamradt laboratory, this hypothesis was tested directly by repeating the immunization strategy outlined above, but this time substituting OspA for HCgp-39, the outer surface protein A derived from the tick-borne spirochete *Borrelia burgdorferi*, the causative organism of Lyme disease [34]. This was a relevant model antigen to study because CD4+ T-cell responses to OspA have been implicated in chronic treatment-resistant Lyme arthritis, and the disease is also associated with HLA-DR4 [35–37]. Large numbers of HLA-DR4-restricted, OspA-specific T-cell hybridomas were generated, and the immunodominant epitopes were identified as before [38].

Two strategies were employed for identifying self-peptide mimics of immunodominant OspA epitopes capable of stimulating OspA-specific T-cell-hybridomas. An example is shown in Fig. 4 for OspA peptide 235–246. In the first strategy, the Swissprotein/TREMBL database was used to define mimics of OspA peptides on the basis of conventional sequence alignment. In the second, a systematic amino acid analysis was undertaken, replacing each residue of the core 9-mer OspA epitope with all naturally occurring amino acids and testing responses of OspA peptide-specific T cells to these synthetic derivative peptides. On the basis of the amino acid substitutions permissive for T-cell responsiveness, a structural ‘supertope’ motif was defined and used to scan the same database. Mimics were identified and synthesized prior to testing on a panel of OspA-specific T-cell hybridomas.

The results were remarkable in several respects [38]. When responses to the ‘best fit’ peptides, identified by either alignment or substitution analyses, were compared on the panel of T cells that responded to the original peptide, reactivity was most frequently observed with peptide mimics fulfilling the supermotif. Intriguingly, many of these peptide mimics, derived from both mouse and human proteins, bore little or no resemblance to the wild-type sequence. Indeed, for some there existed no homologies at any residue. By contrast, few if any peptides identified by conventional sequence alignment stimulated OspA-specific T cells (Fig. 4). This lack of reactivity was analogous to that which I had observed for human HCgp-39 specific T cells when tested on the closely homologous but not identical mouse peptides [22]. These findings support data from other laboratories [39–41], and predict that multiple cross-reactive self-ligands can be identified between immunodominant epitopes of autoantigens and any protein derived from infectious pathogens. They provide direct evidence that T cells apparently highly specific for a given peptide ligand are also broadly cross-reactive to self-peptide ligands, and illustrate that recognition of MHC peptide complexes by TCR is degenerate [41]. The model also indicates that progression across checkpoints 1 and 2 (Fig. 1), namely T-cell activation by cognate antigen and expansion of cross-reactive clones, is almost simultaneous.

Studying the progression through disease checkpoints in the non-susceptible host

The experiments outlined above provided an experimental framework for exploring in the laboratory how the immune system of an RA-non-susceptible host could function in ways that might attenuate inflammatory...
responses in synovial joints. The generation of transgenic mice identical to HLA-DRβ1*0401-expressing mice, with the exception that they express an allele of HLA-DRB1*04 that is not associated with RA (DRβ1*0402), made it possible to address this [27]. A comparative analysis between transgenic lines would also make it possible to examine whether just four amino acid differences, which distinguish DRβ1*0401 from *0402 at codons 86, 69, 70 and 71 (Table 1), were sufficient to alter the molecular interactions between APC and antigen-specific T cells. Closer scrutiny of the sequence and molecular models indicate that these four amino acid changes would have several effects, including a reduction in the size of the P1 peptide-binding pocket (which may influence TCR recognition), and changing the charge preference for peptide residues sitting in pocket 4 from negative to positive [42]. Molecular modelling predicts that gp-39 epitopes presented by DRβ1*0402 should carry small aliphatic hydrophobic residues at P1, and would exclude positively charged residues at P4 [15]. As well as studying the molecular nature of immunodominant peptides processed and presented by DRβ1*0402-expressing APC in vivo, it would also be possible to evaluate whether there was evidence of an attenuated effector response in T cells from transgenic mice carrying the non-associated DRβ1*0402 genotype, and what immunoregulatory mechanisms were in evidence, if any.

Do HLA-DRβ1*0402 molecules present different epitopes to CD4+ T cells in vivo?

Using HCgp-39 as the model antigen, immunization of HLA-DR4-transgenic lines expressing DRβ1*0402 revealed that this molecule, differing from DRβ1*0401 by only four amino acids, presents quite distinct sets of immunodominant peptides of HCgp-39 to CD4+ T cells in vivo [27] (Table 2). As observed for DRβ1*0401 epitopes, these peptides did not carry the expected charged residues at P4. On the other hand, DRβ1*0402, which has a smaller P1 pocket, bound by Val rather than Gly at position β86, favoured binding of peptides with smaller aliphatic residues at P1 (Val or Leu), as predicted from the algorithm of Sinigaglia and Hammer [15]. Because experiments have revealed that there exists in DRβ1*0401 mice a repertoire of T cells capable of recognizing 298–313 peptide [27], one possible explanation for these findings could relate to differences in antigen processing and presentation of this peptide between DRβ1*0401 and *0402-expressing APC. Indeed, collaborative studies with the laboratory of Dr E. Mellins suggest that dependence on HLA-DM for processing of HCgp-39 peptides may explain some of these differences, at least in part [43]. For example, data published by Hall and colleagues [44] since this essay was first submitted have confirmed a relationship between immunogenicity of HCgp-39 peptides and MHC–peptide stability, dictated by the relative sensitivity to HLA-DM editing at pH5.5, the pH of the peptide loading compartment. Regardless of the mechanisms, these data provided evidence that non-associated alleles of HLA-DRB1 profoundly influence the molecular nature of the interactions between TCR and peptide–MHC complexes.

Is the cytokine effector response different for HLA-DRβ1*0402-restricted T cells?

The effects of HLA-DRβ1*0402 on this particular disease checkpoint were addressed by comparing cytokine expression of draining lymph-node T cells from DRβ1*0401 and *0402 mice immunized with HCgp-39, following restimulation with native antigen or the relevant pool of immunodominant epitopes [27]. The results were interesting in several respects. While T-cell proliferation was no different for DRβ1*0401 and *0402-restricted responses, HCgp-39-specific lymph-node T cells from DRβ1*0402-transgenic mice produced significantly less interferon γ (IFN-γ) [27]. On the other hand, DRβ1*0402 T cells specific for OspA produced abundant IFN-γ, indicating that DRβ1*0402 mice did not have an intrinsic defect of IFN-γ production. These data provided the first real clues that one of the consequences of DRβ1*0402 presenting different peptides was to induce a very different profile of proinflammatory cytokine production. The finding of substantially reduced TNF-α levels in DRβ1*0402 cultures is also consistent with this model [27]. The results are also compatible with models which suggest that, unlike disease-associated HLA-DR4 molecules, non-associated HLA-DR4 molecules, such as DRβ1*0402, could provide disease-protective effects by altering the peptides presented to T cells and the cytokines produced as a consequence of these molecular changes (checkpoint 4, Fig. 1). Such a notion was proposed many years ago to account for the sequence differences in diabetes-susceptible and non-susceptible alleles [45]. The data also substantiate the view that overproduction of proinflammatory cytokines early in the evolution of an immune response may be central to the subsequent progression of the inflammatory process (checkpoint 3, Fig. 1). They are consistent with the hypothesis that the proinflammatory cascade is attenuated in the non-susceptible host through the expression of a subset of HLA-DRB1 genes which profoundly influence the molecular nature of CD4+ T cell–APC interactions and immune effector responses (checkpoint 4, Fig. 1).

The chronic phase of inflammation

Much attention has focused on the role of cytokines in the pathogenic events that ultimately result in cartilage destruction and bone erosion [reviewed in 46]. The experiments described above demonstrated that the initial stimulation of T cells by complexes of antigenic peptide and disease-associated, but not non-associated, MHC class II molecules could profoundly influence the expression of proinflammatory cytokines. This cytokine drive may well be central to sustaining the chronic phase of disease, because in mutant mouse models it is possible to induce chronic inflammatory disease in the absence
of functional T and B lymphocytes by sustained over-expression of proinflammatory cytokines in vivo. These mouse models have been highly informative. Nonetheless, for RA patients with an intact adaptive immune system, studying the reciprocal relationship between inflammation and immunity and exploring how chronic overexpression of cytokines might promote T-cell effector responses became a priority.

**T cells are chronically exposed to TNF in synovial joints**

My own studies of how the inflammatory milieu influences the function of chronically activated T cells were undertaken during my PhD studentship in the Feldmann laboratory at the Kennedy Institute of Rheumatology. I began to explore how the chronic inflammatory process influenced the differentiation and effector functions of chronically activated T cells in the belief that the inflammatory environment in established chronic inflammation might be quite different from that which influences the very earliest events of T-helper cell differentiation. At the time, I set out to test the hypothesis that the inflammatory milieu promotes the inflammatory response by enhancing T-cell proliferative and cytokine responses. The results were unexpected, and were partly a consequence of the experimental design. The approach I chose was based on three fundamental experimental observations. First, proinflammatory cytokines, such as TNF and interleukin 1 (IL-1), were expressed and up-regulated at the protein and mRNA levels in synovial tissue from RA patients [47–49]. Secondly, experiments in the laboratory had confirmed that infiltrating mononuclear cells expressed cognate cytokine receptors [50], which were preferentially up-regulated on synovial compared with peripheral blood cells [51], and that ligand colocalized with cells expressing the cognate receptor [52]. This was most comprehensively documented for the prototypic inflammatory cytokine TNF, at a time when the possibility that TNF could be implicated in the chronic phase of RA pathogenesis was only beginning to emerge [53]. Thirdly, I demonstrated for the first time that expression of the naturally occurring TNF inhibitors, the soluble TNF receptor (TNF-R; p55 and p75 sTNF-R), were also substantially increased at the site of inflammation [54]. However, despite levels 3- to 5-fold higher in the synovial joint fluid compared with serum, levels were insufficient to neutralize completely the biological effects of TNF in the joint. Thus, the possibility that activated mononuclear cells, including T cells, were exposed to cytokines such as TNF for prolonged periods at sites of inflammation in vivo was beyond doubt.

**Chronic TNF induces reversible, non-deletional T-cell hyporesponsiveness**

I then set about trying to mimic chronic TNF exposure in an in vitro model, and subsequently in vivo. The results, summarized in Table 3, are derived from an extensive analysis of human and murine T-cell lines and clones in vitro, and from experiments in vivo undertaken in TCR-transgenic mice treated with recombinant TNF or anti-TNF, or after intercrossing to human TNF (hTNF)–globin-transgenic mice, as well as in p55 and p75 TNF-R deficient mice [55–57]. The principle finding—that chronic 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 [58]. Moreover, the p55 TNF-R appeared to be necessary and sufficient for sustained TNF signalling to induce T-cell hyporesponsiveness.

**T cells from patients with RA are hyporesponsive to TCR engagement**

While histological and flow-cytometric analysis of RA synovial T cells and knowledge of the HLA class II associations discussed above have done much to perpetuate the notion that a chronic antigen-driven immune response is central to the disease process [13, 59], functional studies of synovial T cells have continued to perplex workers in this field. The laboratory of Panayi was one of the first to appreciate the potential importance of the finding that proliferative T-cell responses to recall antigens, such as purified protein derivative (PPD), or to mitogens or following cross-linking of the TCR, were dramatically attenuated [50]. Since then a hierarchy has been documented from peripheral blood to synovial tissue, in increasing order of hyporesponsiveness, suggesting not only that loss of T-cell reactivity is acquired, but also that this phenotype may be induced by the inflammatory process itself [61, 62]. According to the results described above, chronic exposure to TNF could provide one possible mechanism for this effect.

**Chronic TNF exposure uncouples proximal TCR signal transduction pathways**

To explore this at the molecular level, a mouse T-cell hybridoma model has been studied in my laboratory, because with this model it is possible to reproduce many of the functional T-cell defects of primary T cells chronically exposed to TNF in vivo, but in the absence of accessory cells [63]. Several lines of experimental evidence suggested that chronic TNF exposure impairs T-cell activation by attenuating TCR signalling pathways [63]. First, T cells chronically exposed to TNF required more peptide for longer periods to commit to IL-2 production, suggesting that the threshold of activation

| TABLE 3. TNF induces non-deletional and reversible T-cell hyporesponsiveness in vitro and in vivo |
|-------------------------------------------------|-------------------------------------------------|
| Chronic exposure to TNF suppresses T-cell activation | Proliferation and cytokine production (Th1 and Th2 cytokines) |
| Dose- and time-dependent | Observed at non-toxic concentrations of TNF |
| Inducible over days | IL-2 and mitogen responses are spared |
| Shedding of soluble TNF-R is impaired | Rapid reversibility in vitro and in vivo on withdrawing TNF |

Data are derived from an extensive analysis of T-cell responses in man and transgenic and mutant mice. For more detailed review see reference 75.

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was dramatically altered. Secondly, cell surface TCR expression was reduced, indicating that not only did chronic TNF uncouple TCR signalling pathways, but also that TNF interrupted assembly of the TCR–CD3 complex and its transport to the cell surface. Thirdly, a detailed biochemical analysis of proximal TCR signalling pathways in TNF-treated T cells confirmed that, through persistent expression and signalling of inflammatory mediators such as TNF, the chronic inflammatory process was capable of uncoupling antigen receptor signalling directly.

In the light of this, it became imperative to explore how chronic TNF targeted the expression of the TCR–CD3 complex, and what effect this might have on downstream signalling pathways (for a schematic of TCR–CD3 assembly and signalling pathways, see references 64 and 65 and Fig. 5). TCR-ζ was one possible target of TNF. Indeed, following closely the kinetics of IL-2 down-regulation, western blotting analysis of whole-cell lysates revealed that chronic TNF exposure suppressed the expression of TCR-ζ in a dose- and time-dependent fashion, while levels of CD3-ε, -γ and -δ, as well as the protein tyrosine kinases ZAP-70, p56Lck and p59Fyn, were not altered [63]. By immunoprecipitation analysis it was possible to demonstrate that cell surface CD3-ε was in fact reduced compared with levels in immunoprecipitates derived from whole cell lysates. This was consistent with a model in which TNF appeared to disrupt the assembly and/or cell surface stability of TCR–CD3 complexes through its effects on TCR-ζ expression [63].

A second unexpected experimental observation provided further evidence that persistent TNF signalling in T cells influenced TCR–CD3 complex assembly and stability at the T-cell surface. Immunoblotting analysis of unstimulated and TNF-stimulated T cells revealed that the expression of the novel transmembrane adapter TRIM (T-cell receptor interacting molecule) was markedly down-regulated by TNF treatment. Closer examination demonstrated that TRIM expression was reduced by TNF before changes in TCR-ζ expression could be detected, and that reconstitution of both TRIM and TCR-ζ 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 [66, 67]. In collaboration with Dr Burkhart Schraven, we demonstrated that

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**Fig. 5.** TCR signal transduction pathways. Polymorphic TCR-αβ chains associate with the invariant chains (CD3-γ, -δ and -ε and TCR-ζ), consisting of non-covalently linked γε and δε heterodimers and disulphide linked ζ–ζ homodimers, which transmit signals inside the cell. TCR ligation leads to the phosphorylation of tandemly arranged tyrosine residues within immunoreceptor tyrosine-based activation motifs (ITAMs) of TCR-ζ chain and CD3-γ, -δ and -ε chains by src family kinases, notably Lck and Fyn. The phosphorylation of adapter proteins, such as p36LAT by ZAP-70 and src kinases, serves as a crucial link between membrane proximal phosphorylation events and the activation of downstream Ras/MAPK (ERK) and calcium signalling pathways. Sustained activation of these pathways is required for transactivation of the transcription factor AP-1 complex and NFAT, leading to IL-2 production, T-cell proliferation and effector responses. LAT, linker for activation of T cells; MAPK, mitogen-activated protein kinase; AP-1, activating protein 1; NFAT, nuclear factor of activated T cells; PLC, phospholipase C; PIP, phosphoinositol phosphate; InsP₃, inositol triphosphate; DAG, diacylglycerol; PKC, protein kinase C; SLP-76, SH2-domain containing leucocyte protein of 76 kDa; Vav, a Rac/Rho-specific guanine nucleotide exchange factor; SOS, son of sevenless.
TRIM plays a role in regulating the cycling of TCR-CD3 complexes at the cell surface [67]. For example, the half-life of TCR-CD3 complexes in stable Jurkat clones overexpressing TRIM is increased. This in turn leads to increased cell surface expression of TCR and enhanced signalling responses, as determined by intracellular calcium mobilization [67]. We can speculate from these experiments that sustained TNF signals in T cells could impair TCR-CD3 assembly not only through its effects on TCR-ζ expression, but also by reducing the half-life of assembled complexes at the cell surface by down-regulating the expression of TRIM. The kinetics of these changes, as well as the precise interactions between TCR-ζ and TRIM, are currently under investigation. Nevertheless, the findings provided a molecular basis for the profound hyporesponsiveness of T cells following TNF stimulation, and predicted that downstream TCR signalling pathways might be significantly attenuated as a result.

Selective uncoupling of downstream TCR signalling pathways by TNF

A comprehensive analysis of signalling pathways in control and TNF-treated T cells has been undertaken to establish to what extent distal TCR signal transduction pathways were attenuated as a consequence of the proximal defects outlined above. As a direct consequence of TCR-ζ phosphorylation being reduced in TNF-treated T cells, downstream signalling events, such as recruitment of ZAP-70 to phospho-TCR-ζ through its SH2 domains and its subsequent phosphorylation, were also impaired [63]. The transmembrane adapter 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 [68, 69] (Fig. 5). LAT and PLCγ1 phosphorylation were substantially reduced in TNF-treated cells, and as predicted intracellular calcium mobilization was also dramatically attenuated [63]. On the other hand, Ras-driven pathways, including activation of the MAPK ERK activation, c-Fos induction and TCR-induced CD69 expression, were preserved [J. Clark, K. Aleksiyadis and A. Cope, unpublished data]. These findings identify a novel molecular basis for T-cell hyporesponsiveness. They are of interest because attenuation of intracellular calcium mobilization and preservation of Ras/ERK (extracellular signal-regulated kinase) signalling is the reverse of the aberrant TCR signalling pathways defined in anergic T cells by other laboratories, whereby calcium responses are spared and Ras/ERK signals are attenuated [70, 71].

TCR signalling is also defective in RA synovial T cells

Impaired TCR signalling has been well documented in RA synovial T cells. Down-regulation of TCR-ζ chain expression and abnormal recruitment and phosphorylation of p36 LAT have also been documented [72, 73]. Significantly, calcium mobilization is also reduced in peripheral blood as well as synovial T cells from patients with RA [74, 75], while unpublished data indicate that Ras activation is up-regulated in synovial T cells (C. Verweij, personal communication). On the basis of our own studies, TNF could be one of several factors, including other cytokines or reactive oxygen species, responsible for the uncoupling of TCR signals in chronic inflammatory diseases such as RA in man [55, 76].

Studying T-cell hyporesponsiveness in the mouse: lessons from in vivo models

The precise significance of T-cell hyporesponsiveness in the context of chronic inflammatory disease in vivo is not clear. Nevertheless, the distinctive cell phenotypes shared by RA synovial T cells and T cells chronically exposed to TNF in vitro or in vivo (summarized in Table 4) have made it possible to draw some tentative conclusions based upon results derived from experiments in the mouse. I have recently reviewed the possible contributions that hyporesponsive T cells could make to the pathogenesis of autoimmune disease [32, 76, 77]; these are illustrated in Fig. 6. Several points are worthy of note. Jacob and McDevitt [78] first demonstrated that TNF therapy was beneficial in a murine model of lupus. Similar results followed in the NOD mouse [79, 80], a model of spontaneous type I diabetes, and subsequently

TABLE 4. Chronic exposure of T cells to TNF induces a phenotype resembling CD4+ T cells derived from inflamed RA synovial joints

<table>
<thead>
<tr>
<th>Up-regulation of activation antigens</th>
<th>Induction of proliferative hyporesponsiveness</th>
<th>Suppression of T-cell cytokine production</th>
<th>Repression of CD28 gene transcription</th>
<th>Uncoupling of proximal TCR signal transduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factors contributing to disease promotion</td>
<td>Reduced T cell clonal expansion</td>
<td>Suppressed autoreactive T cell responses</td>
<td>Suppressed Th1 effector functions</td>
<td>Chronic inflammation</td>
</tr>
<tr>
<td>Reduced Th2 immuno-regulatory function</td>
<td>Impaired activation induced cell death</td>
<td>Depressed immunity to foreign pathogens</td>
<td>Impaired anti-tumour immunity</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 6. Possible mechanisms through which hyporesponsive T cells could contribute to the pathogenesis of chronic inflammatory arthritis. Hyporesponsive T cells could reflect an adaptive response aimed at suppressing T-cell autoreactivity. According to this model, T cells would play little or no role in promoting disease, since hyporesponsiveness constitutes a protective response (left panel). Alternatively, impaired T-cell activation could promote and perpetuate the chronic inflammatory response, through loss of tolerance mechanisms that require intact TCR signalling pathways (right panel). Host defence would be compromised through similar mechanisms.
in studies of experimental autoimmune encephalomyelitis [81], the murine counterpart of multiple sclerosis in man. My own studies using similar treatment protocols, but in a TCR-transgenic mouse model, demonstrated conclusively that TNF could suppress T-cell effector responses in vivo, either when TNF was administered intraperitoneally or when TCR-transgenic mice were crossed to human TNF–globin transgenic mice [57]. The finding that neutralizing TNF in healthy TCR-transgenic mice enhanced T-cell responses provided the first evidence that physiological expression of this cytokine could suppress T-cell responses in vivo. More recently, the outcome of disease progression in autoimmune-prone mice carrying null mouse TNF or TNF-R (knockout) genes has substantiated the view that TNF has potent immunomodulatory effects in vivo [81–84]. These data point to the possibility that the suppressive effects of chronic TNF are protective, perhaps reflecting an adaptive response to inflammatory signals which attenuate pathogenic T-cell effector responses [57, 76] (Fig. 6, left panel).

**Studying T-cell hyporesponsiveness in man: lessons from the clinic**

Back in 1992, I was provided with a unique opportunity to test in patients whether TNF could suppress T-cell activation in man. At this time, a cohort of RA patients with active disease had been recruited to an open-label trial of anti-TNF monoclonal antibody therapy [infliximab (Remicade®)] for the first time. By studying peripheral blood T-cell proliferative responses to recall antigens and mitogens before and after treatment, I found that T-cell responses were dramatically restored [55]. These findings were entirely in keeping with the in vitro data [55], and have since been reproduced by several groups [85–87], including a more recent study of T-cell responses to influenza haemagglutinin peptide and collagen II in RA patients treated with the p75 TNF-R–Fc fusion protein etanercept (Enbrel®) [88]. Despite this enhanced T-cell reactivity, multicentre trials of anti-TNF have demonstrated conclusively a consistent and rapid improvement in clinical parameters in ~70% of RA patients [89–91]. Equally striking clinical responses to anti-TNF have been documented in inflammatory bowel disease, particularly Crohn’s disease [92, 93]. These therapeutic responses have prompted an evaluation of TNF blockade in psoriatic arthritis, ankylosing spondylitis and vasculitis.

While it has been postulated that the clinical response reflects the potent anti-inflammatory properties of anti-TNF, including effects on cell trafficking and angiogenesis [94, 95], the sustained clinical response to a single infusion of anti-TNF for periods that extend far beyond the time point at which antibody is cleared from the body [89] suggests that anti-TNF may induce lasting immunomodulatory effects (Fig. 6, right panel). We are currently exploring in the laboratory the possibility that, through restoring thresholds of T-cell activation, the immune system is reset, regaining the regulatory functions of T cells which in the non-susceptible host effectively suppress inflammatory responses. By restoring the integrity of TCR signal transduction pathways, autoreactive T cells would also acquire the capacity to undergo activation-induced cell death. The finding that gut CD4+ lymphocytes from patients with Crohn’s disease undergo apoptosis following anti-TNF therapy is consistent with this model [96]. This sudden increase in apoptosis might also explain, at least in part, the development of antinuclear antibodies in ~8% of patients treated with anti-TNF [97]. On the other hand, the concept that restoring T-cell responses would enhance host responses to foreign pathogens, as well as anti-tumour immunity, is more obvious. In disease-susceptible individuals, therefore, sustained overexpression of TNF could contribute to disease pathogenesis through effects which include attenuation of TCR signalling. This might lead to the failure of immunoregulatory mechanisms critical for maintaining peripheral tolerance, such as activation-induced cell death and immune deviation (Fig. 6, right panel). According to the proposed model, TCR signalling and, by inference, immunoregulation would be predicted to be intact in non-susceptible individuals (checkpoint 4, Fig. 1).

**Is a shift from ‘antigen mode’ to ‘inflammation mode’ characteristic of effector T cells in chronic inflammatory disease?**

As TCR hyporesponsiveness in chronic inflammation appears to correlate with disease severity and or chronicity regardless of immunosuppressive therapy [55, 61], it is difficult to reconcile the long-held belief that T-cell effector responses in inflamed joints are exclusively antigen-driven. Rather, data suggest that during the chronic phase of the disease process it may be the cytokine milieu that sustains and maintains pathogenic T cells [98, 99]. According to this model there is a gradual decline of antigen-induced proliferative responsiveness as cytokine drive increases (Fig. 7). Nevertheless, antigen drive and cytokine drive are inextricably linked in the sense that the initial antigen response initiates both a cytokine cascade and the acquisition of cytokine responsiveness through up-regulation of cytokine receptors, while T-cell hyporesponsiveness arises through subsequent sustained expression of inflammatory cytokines (Fig. 7).

How could hyporesponsive T cells contribute to the effector phase of inflammation? Or are they passive bystanders? One possible explanation lies in the observation that depletion of hyporesponsive T cells from RA synovial mononuclear cell cultures significantly reduces the expression of proinflammatory cytokines [100]. Evidence suggests that this process of activation may be mediated by cell-to-cell contact, by as yet poorly defined receptor–ligand interactions [101–103]. Failure of synovial joint T cells from RA patients to undergo apoptosis could further facilitate cell-to-cell signals and perpetuate the inflammatory response [98]. Perhaps B-cell help and autoantibody production is enhanced through similar cell-to-cell mechanisms [reviewed in 104]. In order to
address this issue specifically, we have recently undertaken gene expression profiling of control and TNF-treated mouse T cells. Preliminary data suggest there is a TNF-induced gene expression signature which, in vivo, would be predicted to favour Th1 cell differentiation [induction of interferon regulatory factor-1 (IRF-1), IL-12Rβ1 and IFN-γR genes], recruitment of T cells to inflamed joints [fucosyltransferase VII, interferon-gamma-inducible protein 10 (IP-10) and RANTES (regulated upon activation, normal T-cell expressed and secreted cytokine)], effector responses initiated through cell surface ligands such as RANK (receptor activator of nuclear factor κB) ligand and cell survival (induction of anti-apoptotic genes cIAP-2, A20 and Bis). These may turn out to be important results because if the data can be reproduced in vivo they imply that hyporesponsive synovial T cells are not inert, but have the potential to become potent effector cells through dominant cytokine-driven signal transduction pathways. Selective uncoupling of TCR signalling and sparing of antigen-specific Ras/ERK activation would contribute significantly to this phenotype by promoting cell survival and the persistence of activated cells in inflamed joints.

Concluding remarks

Confirming unequivocally that hyporesponsive T cells are capable of promoting the inflammatory process in RA is a priority because an understanding of the precise mechanisms involved could provide a molecular basis for testing hitherto unexplored immunotherapeutic strategies. Studying the effects of suppressing the Ras/ERK pathway is just one example. To date, the assumption has been that T cells in RA are harmful, because effector responses are sustained by chronic activation by tissue-specific antigens presented by disease-associated HLA class II molecules (Fig. 1). The assumption that synovial T cells are harmful is probably correct, but perhaps for the wrong reasons. For example, T-cell-targeted strategies have focused almost exclusively on depressing T-cell function further. The results have been disappointing [105, 106], perhaps because the calcium ≡ calcineurin pathway and transactivation of nuclear factor of activated T cells (NFAT) are already profoundly depressed in cytokine-activated T cells. However, results from several laboratories, including my own, suggest that therapeutic strategies aimed at restoring the function of subsets of T cells should now be considered. A major challenge will be to identify which specific T-cell subsets in vivo are hyporesponsive. For example, if these subsets include regulatory T cells, such as IL-10-producing Tr1-like CD4+ cells or IL-16-producing CD8+ T cells [107, 108], restoring TCR responsiveness could suppress the inflammatory activities of offending effector T-helper cell subsets, macrophages and fibroblasts.

This therapeutic approach is entirely in line with the thesis proposing that susceptibility to autoimmunity arises through failure of the adaptive immune system to regulate the inflammatory response. In the short term, restoring T-cell responsiveness towards ‘normal’ (but not beyond) could be achieved by a combination of immunotherapeutic approaches targeting the peptide or altered peptide ligand specificities of regulatory T cells (which could be determined in HLA class II-transgenic mice as described above) together with anti-TNF. This approach would provide anti-inflammatory cover, while at the same time enhancing responsiveness to the therapeutic peptide by restoring TCR signalling pathways. A detailed knowledge of the specific TCR signalling defects could provide clues as to how best to monitor such therapy, and how to avoid rebound T-cell hyper-reactivity and its inevitable consequences, systemic autoimmune disease and acute cytokine release syndromes. Anti-TNF in combination with non-depleting anti-CD4 or anti-CD3 monoclonal antibody could have similar beneficial therapeutic effects [109]. If, on the other hand, this hypothesis is incorrect and T-cell hyporesponsiveness turns out to be beneficial to the disease process, then a more precise understanding of how the inflammatory process uncouples signalling pathways in cytokine-activated T cells would greatly facilitate the development of novel immuno-suppressive agents.

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