T-cell anergy and peripheral T-cell tolerance

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The discovery that T-cell recognition of antigen can have distinct outcomes has advanced understanding of peripheral T-cell tolerance, and opened up new possibilities in immunotherapy. Anergy is one such outcome, and results from partial T-cell activation. This can arise either due to subtle alteration of the antigen, leading to a lower-affinity cognate interaction, or due to a lack of adequate co-stimulation. The signalling defects in anergic T cells are partially defined, and suggest that T-cell receptor (TCR) proximal, as well as downstream defects negatively regulate the anergic T cell's ability to be activated. Most importantly, the use of TCR-transgenic mice has provided compelling evidence that anergy is an *in vivo* phenomenon, and not merely an *in vitro* artefact. These findings raise the question as to whether anergic T cells have any biological function. Studies in rodents and in man suggest that anergic T cells acquire regulatory properties; the regulatory effects of anergic T cells require cell to cell contact, and appear to be mediated by inhibition of antigen-presenting cell immunogenicity. Close similarities exist between anergic T cells, and the recently defined CD4⁺CD25⁺ population of spontaneously arising regulatory cells that serve to inhibit autoimmunity in mice. Taken together, these findings suggest that a spectrum of regulatory T cells exists. At one end of the spectrum are cells, such as anergic and CD4⁺CD25⁺ T cells, which regulate via cell-to-cell contact. At the other end of the spectrum are cells which secrete anti-inflammatory cytokines such as interleukin 10 and transforming growth factor- β . The challenge is to devise strategies that reliably induce T-cell anergy *in vivo*, as a means of inhibiting immunity to allo- and autoantigens.

Keywords: T cells; tolerance; anergy; regulation

1. INTRODUCTION

Intrathymic deletion of thymocytes with high affinity for self-antigen cells plays a crucial role in contracting the autoreactive T-cell repertoire. However, this is manifestly an incomplete process. Not all self-proteins are effectively presented in the thymus, including those that are expressed well after the bulk of the T-cell repertoire has been formed, and it is relatively easy to detect autoreactive T cells following immunization with self-antigens. For this reason mechanisms of regulating peripheral T cells with unwanted specificity are crucial to survival. There are several mechanisms of peripheral T-cell unresponsiveness including ignorance, deletion by apoptosis and cytokine-mediated regulation. The topic of this review is a further mechanism, T-cell anergy. Data will be highlighted which suggest that the induction of T-cell anergy is an important contributor to peripheral T-cell tolerance, and that anergic T cells are not passive, but may play an important role as regulatory cells.

Central to the physiology of T lymphocytes is the fact that ligation of the antigen-specific receptor can have profoundly different outcomes, depending upon the nature of the non-cognate signals from the antigen-presenting cell (APC). The concept that lymphocytes require more than one signal for full activation was first articulated by Bretscher & Cohn (1970), although it was some years before any data were given in support of this

idea. Working in murine transplantation models, involving pancreatic islets and thyroid grafts, Lafferty & Gill (1993) evolved the concept that certain cell types and tissues provided 'co-stimulation' while others did not. Only tissues that contained co-stimulation-positive cells induced rejection when transplanted across a major histocompatibility complex (MHC) barrier. These studies paved the way for more detailed analyses with T-cell clones, carried out by Jenkins & Schwartz (1987) and Quill & Schwartz (1987), who used as APCs either chemically fixed cells or planar membranes impregnated with MHC class II molecules and antigenic peptide. Both of these artificial 'APCs' not only failed to induce proliferation by the T-cell clones, but also induced antigen-specific unresponsiveness, or anergy. These data opened up a field of investigation on the topic of T-cell anergy, as reviewed below.

2. DEFINITIONS

The term anergy was coined by Nossal & Pick (1980) to describe the unresponsive state that was induced in B cells following the injection of soluble proteins *in vivo*. They observed that the antigen-specific B cells were present, but were refractory to subsequent activation by antigen or mitogen. The first observations of antigen-induced T-cell proliferative unresponsiveness were made with human T-cell clones following culture with antigen in the absence of added APCs (Lamb *et al.* 1983). Although initially interpreted to be the effect of free

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antigen, anti-MHC class II antibody-blocking experiments soon revealed that the unresponsive state was due to the presentation of antigen by the MHC class II molecules expressed by the T cells themselves (Lamb & Feldmann 1984). Since that time the term anergy has been used to describe a variety of forms of unresponsiveness, induced in a variety of ways, and mediated by diverse mechanisms. This has led to some confusion, and scepticism as to whether anergy was a useful or informative term.

The definition of anergy that we favour is the following: 'a state of long-lasting, partial or total unresponsiveness induced by partial activation'. This excludes unresponsiveness due to 'blindness' or 'partial sightedness' resulting from temporary T-cell receptor (TCR) modulation, and the refractory state that can be seen when T cells are prematurely exposed to activating stimuli too soon after their last antigen encounter. The large majority of reports of anergy have described the inhibition of interleukin 2 (IL-2) secretion by T cells that are normally capable of secreting IL-2 upon activation. However, anergy has also been described in T helper type 2 (Th2) cells, in which the secretion of the key signature cytokine IL-4 is unaffected, but the ability of the T cells to proliferate in response to IL-4 is inhibited (Sloan-Lancaster *et al.* 1994b). The common theme in these examples is that anergy refers to the inability of T cells to produce or respond to proliferative signals. It is important to note that the unresponsiveness is often partial. This is well illustrated by the observations that T helper type 0 (Th0) cells continue to secrete IL-4 after the induction of anergy (Mueller *et al.* 1991) and T cells with the capability of secreting IL-2 and mediating cytotoxicity retain their cytotoxic potential after being rendered anergic even though IL-2 secretion is shut down (Otten & Germain 1991).

Having attempted to provide a universal definition of anergy, it may well be that different 'levels' of anergy exist (Arnold *et al.* 1993). This is illustrated by the finding that anergy can sometimes be reversed by culture in exogenous IL-2 leading to several rounds of cell division (Beverly *et al.* 1992), while in other systems this has been found not to be the case (Groux *et al.* 1996). These differences are probably explained by differences in the state of activation of the starting T-cell populations, and by differences in the partial activation signals that were used to induce anergy. As the intracellular signalling events that accompany anergy become better defined, as discussed below (§4), it may be possible to explain these differences.

3. MECHANISMS OF ANERGY INDUCTION *IN VITRO*

There are at least four distinct sets of circumstances that have been observed to induce T-cell anergy *in vitro*; these are represented schematically in figure 1, and will be discussed in turn.

(a) *TCR ligation in the absence of full co-stimulation*

This is the most extensively studied set of conditions that leads to T-cell unresponsiveness. The first observations of this kind were made in the artificial, but very precisely defined, system of Quill & Schwartz (1987). They exposed mouse T-cell clones to artificial lipid

Figure 1. The different circumstances that have been observed to induce T-cell anergy *in vitro* are illustrated. (a) Recognition without co-stimulation. (b) Recognition of altered peptide-MHC ligand. (c) Full stimulation without IL-2R signalling or division. (d) T-T antigen presentation. Abbreviations: ICAM-1, intercellular adhesion molecule 1; LFA-3, leucocyte function-associated molecule 3.

bilayers, coated onto glass cover-slips, and impregnated with H2-E^k molecules together with the appropriate peptide of pigeon cytochrome *c*. They noted that the T cells increased in size, secreted small amounts of IL-3, but failed to secrete IL-2 or proliferate. In contrast, a T-cell hybridoma with the same specificity secreted IL-2 in response to the surrogate APCs. Most importantly, the T-cell clone failed to proliferate when rechallenged one week later with peptide-pulsed competent APCs, in other words it had been rendered 'anergic'. At the time, the nature of the missing 'co-stimulatory' molecules was completely unknown; however, the identity of one of the key molecules followed four years later when B7 (CD80) was cloned (Freeman *et al.* 1989) and found to ligate the T-cell-surface molecule CD28 (Linsley *et al.* 1991a). Definition of this major co-stimulatory pathway spawned an era of experimentation *in vitro* and *in vivo* during which the consequences and exploitability of co-stimulation-deficient antigen presentation were explored. This era has extended up to the present day, in that some of the first clinical trials of co-stimulatory blockade in the context of autoimmune disease and transplantation are currently taking place (Guinan *et al.* 1999). The generation of a fusion protein, cytotoxic T-lymphocyte antigen 4 immunoglobulin (CTLA-4-Ig), composed of the second receptor for B7 molecules on T cells, namely CTLA-4 (CTLA-4 function is discussed later (§ 3(f))), fused to the Fc portion of Ig, facilitated many of these experiments.

Most of the observations of anergy in response to TCR ligation in the absence of full co-stimulation have involved established mouse or human T-cell clones (Schwartz 1996). In contrast, naive T cells appear not to be susceptible to the induction of anergy under most of these conditions *in vitro* (Davis & Lipsky 1993; Sagerstorm *et al.* 1993; Hayashi *et al.* 1998). One exception to this generalization was noted when plastic-immobilized anti-CD3 monoclonal antibody (mAb) was used as the means of ligating the TCR on naive T cells (Williams *et al.* 1992; Wolf *et al.* 1994; Chai & Lechler 1997). Although this may not reflect conventional antigen presentation *in vivo*, it may resemble the events accompanying exposure to bacterial endotoxins (superantigens, SAgS) that deliver a strong signal through the TCR-CD3 complex to a large fraction of the T-cell repertoire. In this context it is noteworthy that several of the descriptions of anergy *in vivo* have involved the injection of bacterial SAgS, as discussed in detail below (§ 5).

(b) Exposure to peptide partial agonists

In the process of screening peptides for those with improved binding characteristics to MHC molecules, Sette and colleagues (De Magistris *et al.* 1992) discovered that some analogues, usually differing by a single amino acid from the parent peptide, acted as antagonists. These antagonists induced no detectable signalling to the T cell, and had no effect on subsequent T-cell reactivity. Soon after this, Sloan-Lancaster *et al.* (1993) described amino-acid-substituted peptide variants that not only failed to induce T-cell proliferation, but that induced T-cell anergy. In contrast with the first conditions that were discovered to induce anergy, the effect of these peptide partial agonists was observed even when the APC provided full co-stimulation. Similar observations were made by

Germain and colleagues (Racioppi *et al.* 1993) using transfectants expressing amino-acid-substituted MHC class II molecules as APCs. As the nature of TCR-CD3-transduced signals was elucidated it became clear that these altered peptide ligands (APLs), or altered MHC molecules, induced a characteristic pattern of early signalling events, suggestive of partial activation (Sloan-Lancaster *et al.* 1994; Madrenas *et al.* 1995). This will be discussed further below (§ 4).

More recently, the technique of surface plasmon resonance has been applied to the study of TCR interactions with native and altered peptides. The general observation has been that the TCR has significantly faster off-rates from partial agonists, compared with native peptides (Lyons *et al.* 1996). Several explanations have been proposed to link these observations with the induction of anergy. Our own interpretation was that there is a fast-kinetic and a slow-kinetic event following TCR ligation. The fast off-rate interaction with the APL would allow the fast, but not the slow, reaction to occur (Lord *et al.* 1999). Native peptide would be bound for sufficient time for both reactions to proceed. The induction of anergy was consequent upon the fast reaction products that mediated inhibitory effects in the cell. Either the slow reaction product led to the neutralization of the inhibitors, or the effects of the slow reaction products dominate, allowing the cell to undergo IL-2-driven division and thereby diluting the inhibitors that would otherwise maintain the unresponsive state.

There has been considerable interest in the possibility that APLs could be used therapeutically (Frasca *et al.* 2000). However, their potential is limited by the fact that a peptide that acts as an APL for one T-cell clone-TCR may well be an agonist or entirely neutral for other clones with the same antigen specificity. Therapeutic use of APLs in autoimmune disease or in transplantation would require, therefore, that the T cell affected by the APL could regulate other T cells with the same autoantigen or alloantigen specificity. The finding that certain viral escape mutants encode APL, in place of a CTL epitope, may be taken to indicate the potential of this approach (Klenerman *et al.* 1994; Bertoletti *et al.* 1994).

(c) Full signalling without IL-2 receptor (IL-2R)-driven cell division

The importance of cell division in the maintenance of T-cell reactivity was demonstrated by experiments in which mouse T-cell clones were cultured with co-stimulation-positive antigen-pulsed APCs, but were prevented from dividing by the addition of anti-IL-2 and anti-IL-2R antibodies (DeSilva *et al.* 1991). This observation could be explained in two ways. First, it could be that cell division is necessary to avoid unresponsiveness. This would be consistent with the suggestion that inhibitors of activation, 'anergy proteins', are invariably generated in the context of T-cell activation, but that the T cell escapes from their inhibitory effects by the dilution that results from division. A second possible explanation for the anti-IL-2R result is that IL-2R signalling is itself important in maintaining T-cell responsiveness. These possibilities have recently been addressed using a drug, rapamycin, that blocks IL-2R signalling and prevents transition from the G1 to the S phase of the cell cycle.

T cells were cultured with immobilized anti-CD3 and anti-CD28 in the presence or absence of rapamycin. Despite TCR-CD3 and CD28-mediated signals the cells cultured in the presence of rapamycin became anergic (Powell *et al.* 1999). The additional finding in this study was that cell-cycle arrest by addition of hydroxyurea did not have the same effect. This suggests that IL-2R-mediated signals, independent of the induction of G1 to S phase transition, prevent the induction of anergy.

(d) *TCR ligation in the presence of IL-10*

IL-10 is clearly an important regulatory cytokine, and has been implicated in some models of transferable T-cell tolerance. The first connection between T-cell anergy and IL-10 was made by Groux *et al.* (1996) who noted that stimulation of human CD4⁺ T cells in the presence of IL-10 induced a state of profound unresponsiveness that could not be reversed with IL-2. These findings may be explained by later observation that stimulation of T cells in the presence of IL-10 favoured the emergence of IL-10-secreting T cells. Such cells have regulatory properties, and have been labelled as Tr1 (regulatory) cells (Groux *et al.* 1997). In retrospect, the description of the IL-10 effect could be reinterpreted as 'immune deviation' rather than as the induction of anergy. However, a spectrum of non-responsive T cells appears to exist, and the distinction between anergy and immune deviation may be semantic.

(e) *T-T antigen presentation*

The earliest observations of T-cell anergy arose from the culture of human T-cell clones with antigen in the absence of APCs, as described above (§ 2) (Lamb *et al.* 1983; Lamb & Feldmann 1984). Initially this was thought to require high antigen concentrations; however, it was subsequently shown to occur in the presence of the same antigen concentration that induced optimal proliferation when presented by professional APCs (Sidhu *et al.* 1992). This is an anomalous finding, in that activated human T cells, as used in these experiments, not only express high levels of MHC class II molecules, but also high levels of B7 family molecules (Wyss-Coray *et al.* 1993; Azuma *et al.* 1993; Sansom *et al.* 1993). It has been suggested that the B7 molecules expressed by human T cells are non-functional due to altered glycosylation (Hollberg *et al.* 1997). However, we find that activated human T cells are able to stimulate resting T cells in the allogeneic mixed lymphocyte reaction, suggesting that their B7 molecules are fully functional (Lombardi *et al.* 1994a). The molecular mechanisms responsible for this form of anergy remain to be adequately explained, although some information is available. For example, it appears that mitogen-activated protein (MAP) kinase activation proceeds normally suggesting that the Ras pathway is normally activated (G. Lombardi and R. Lechler, unpublished data). In contrast, the calcium (Ca²⁺) flux induced by T-T presentation is markedly reduced compared with that observed following antigen presentation by competent APCs (Lombardi *et al.* 1996). These observations suggest that TCR-CD3 signalling is altered in some way. The possibility that CTLA-4 (CD152) ligation contributes to the effects of T-T responses has not been fully explored; given that CD152 signalling can alter CD3-transduced signals (Lee *et al.*

1998), this is a reasonable candidate explanation for this unusual form of anergy.

(f) *Role of CTLA-4 in the induction of anergy*

It was discovered in 1991 that the long-known T-cell activation antigen, CD152, was a second ligand for the B7 family of molecules (Linsley *et al.* 1991b). It was soon apparent that CD152 had a negative regulatory role, with almost opposite effects to those of CD28 (Walunas *et al.* 1994; Krummel & Allison 1995). The most startling results were from mice in which the CD152-encoding gene was inactivated. These mice died at approximately three weeks of age with overwhelming lymphocyte accumulation (Waterhouse *et al.* 1995; Tivol *et al.* 1995).

The involvement of CD152 in anergy induction was first suggested by experiments in DO11.10 TCR-transgenic mice (Perez *et al.* 1997). It was noted that injection of soluble ovalbumin, the antigen for which the transgenic T cells were specific, induced a cohort of unresponsive cells. If anti-CD152 antibody was co-injected with the ovalbumin, the induction of anergy was prevented. Later Walunas & Bluestone (1998) also showed that CD152 blockade prevents Staphylococcus Enterotoxin B-induced T-cell anergy *in vivo*. CD152-B7 interactions also appear to influence the development of transplantation tolerance. Administration of anti-CD152 mAb to murine recipients of skin allografts completely reversed the tolerance induced by thymectomy, anti-CD154 (CD40 ligand) antibody and donor-specific blood transfusion (DST) (Zheng *et al.* 1999). Similar findings were reported in a mouse heart graft model in which long-term graft acceptance was achieved by recipient pretreatment with DST in conjunction with CTLA-4-Ig; administration of anti-CD152 at the time of transplantation significantly reduced graft survival time (Judge *et al.* 1999). Whether anti-CD152 mAb in these models is inhibiting the induction of anergy, deletion or the emergence of regulatory cells has yet to be determined.

Contradictory results have also been reported. In a nasal tolerance model, anti-CD152 did not reverse the antigen-induced unresponsiveness; however, it is not clear that the mechanism of unresponsiveness was T-cell anergy (Tsitoura *et al.* 1999). In an *in vitro* system Frauwirth *et al.* (2000) observed that T cells from CD152-deficient mice were equally susceptible to the induction of anergy by anti-CD3 antibody.

(g) *Anergy in CD8⁺ T cells*

Despite the large amount of data documenting the existence of anergic CD8⁺ T cells *in vivo*, the origin and functional status of these T cells have yet to be clearly defined. The central functional defect of classical anergy as described for CD4⁺ T cells is the lack of autocrine proliferation upon antigenic rechallenge. However, this criterion cannot be applied to functionally competent CD4-dependent CD8⁺ T cells, which require helper-derived IL-2 for their expansion and differentiation. Thus a distinction must be made between anergy induction in helper-dependent and helper-independent CD8⁺ T cells. *In vivo* studies have suggested that helper-independent CD8⁺ T-cell expansion occurs at an early stage of the immune response as autocrine IL-2 production is not sufficient to support a sustained CD8⁺ T-cell response,

which becomes gradually dependent on CD4⁺ T-cell help (Kirberg *et al.* 1993; Matloubian *et al.* 1994; Bennett *et al.* 1997; Cardin *et al.* 1996; Ossendorp *et al.* 1998).

In keeping with what has been described for CD4⁺ T cells, anergic helper-independent CD8⁺ T cells have been shown to be unable to produce IL-2 and undergo cell division in response to subsequent stimulation. This form of CD8⁺ T-cell anergy has been shown to be induced by cognate recognition of immature dendritic cells (DCs) (Steinbrink *et al.* 1999) or IL-10-treated APCs (Groux *et al.* 1998), and by T–T antigen presentation (Chai *et al.* 1998). In addition, CD8⁺ T-cell anergy has been described as an end-point in the development of fully stimulated CD8⁺ T cells (Deeths *et al.* 1999). This form of activation-induced non-responsiveness (AINR), which is reversible by exogenous IL-2, has been hypothesized to be a physiological mechanism to convert early helper-independent into helper-dependent CD8⁺ T-cell responses. Finally, CD28 downregulation has been correlated with functional hyporesponsiveness in CD8⁺ T cells from human immunodeficiency virus-infected individuals (Lloyd *et al.* 1997). In most studies of CD8⁺ T cells the anergic state could be reversed by the addition of IL-2, and did not affect interferon- γ (IFN- γ) production.

It is more difficult to establish whether an anergic state can be induced in the helper-dependent CD8⁺ T-cell pool, and even harder to establish criteria to define this state, as these cells are incapable of autocrine proliferation. The ‘inability to expand and differentiate following antigenic rechallenge in the presence of CD4⁺ T-cell help’ could be an appropriate definition, but to date no evidence exists to indicate that this occurs.

A provocative suggestion from Mescher (Deeths *et al.* 1999), based on his observations of activation-induced loss of IL-2 secretion by CD8⁺ T cells, is that helper-dependent CD8⁺ T cells have undergone AINR and are anergic, but can clonally expand and differentiate in response to CD4⁺ T-cell-derived IL-2.

(h) Relationship between anergy and apoptosis

Until recently the majority view was that anergy was functionally equivalent to a cell’s ‘last gasp’ before admitting defeat and dying by apoptosis. Because it was possible, *in vitro* at least, to maintain cells in this ‘pre-terminal’ state, the anergic cell could be studied in detail. Although there is clear overlap between anergy and apoptosis it is now clear that they are distinct outcomes of partial activation of a T cell. This was investigated using human T-cell clones rendered unresponsive following T–T peptide presentation, as described above (§3(e)) (Hargreaves *et al.* 1997). Some clones emerged from these cultures live but unresponsive, i.e. anergic, while other clones underwent apoptosis such that only 10% of T cells survived. The distinction between these two patterns of response appeared to be determined by sensitivity to Fas-mediated death, in that addition of a neutralizing anti-Fas antibody to the apoptosis-prone clone rescued it from death. Interestingly, the rescued clone was then found to be profoundly anergic. The molecular basis for the difference between these sets of clones was not defined.

Similar findings have been observed using anti-CD3 mAb stimulation of naive murine T cells, in the absence of APCs (Chai & Lechler 1997). Culture of naive T cells

with immobilized anti-CD3 antibody is commonly associated with apoptotic death in *ca.* 40% of T cells; the surviving T cells emerge with a classically anergic phenotype, being hyperresponsive to exogenous IL-2, but unable to undergo autocrine proliferation in response to full activation stimuli. The difference between the 40% that undergo apoptosis and the remainder that do not is unclear. Similar findings were reported by Frauwirth *et al.* (2000), who noted that T cells from mice transgenic for Bcl-xL under the *lck* promoter were equally susceptible to the induction of anergy by anti-CD3 antibody.

4. INTRACELLULAR SIGNALLING EVENTS ACCOMPANYING ANERGY INDUCTION

A large body of data has accumulated concerning the intracellular mechanisms responsible for, or correlated with, T-cell anergy. The material used for these analyses included mouse and human T-cell clones, and T cells rendered anergic *in vivo* by SAg injection. The studies can be clustered into four groups: those that found differences in early TCR-linked signalling events, those that found altered regulation of Ras, those that found alterations in expression, activity, or DNA binding of the transcription factor complex activator protein 1 (AP-1), and finally those that detected negative regulators of IL-2 gene transcription. The background to these observations is a consistent finding that the induction of anergy is dependent upon Ca²⁺-dependent signals. In keeping with this, treatment of T-cell clones with ionomycin in the absence of any other signals is one way to induce anergy (Jenkins *et al.* 1987), and the addition of cyclosporin A prevents the induction of anergy (Quill & Schwartz 1987). The latter observation may have significant implications for the choice of immunosuppressive drug regime to combine with tolerance-promoting strategies in transplant recipients. The requirement for a Ca²⁺ flux illustrates the fact that the induction of anergy in T cells is an active process that requires antigen recognition and partial activation.

Taking the four sets of findings concerning altered signalling in anergic T cells in turn, the most upstream alterations that have been noted have involved TCR–CD3 complex-proximal events. Two groups have reported altered activation of two Src-family kinases, p56^{lck} and p59^{lyn}. Quill *et al.* (1992) observed a decrease in the level of p56^{lck} and a corresponding increase in the amount of p59^{lyn}. Gajewski *et al.* (1994) noted increased activity of the p59^{lyn} kinase in anergic cells; they also noted that anergic T cells had a blunted Ca²⁺ flux upon antigen stimulation, further suggesting altered TCR–CD3-transduced signalling. Both groups noted, possibly as a consequence of this, an altered pattern of phosphorylation of several proteins in cell lysates of anergic, compared with normal, T cells following antigen rechallenge (Cho *et al.* 1993; Bhandoola *et al.* 1993; Gajewski *et al.* 1994). Quill’s group extended these observations into an *in vivo* system using an SAg stimulus. The only question mark concerning these findings is whether they were related to the use of immobilized anti-CD3 antibody or an SAg to induce the anergic state. The controls for these experiments were resting unmanipulated T cells; the better control for the *in vitro* experiments would have been co-immobilized

anti-CD3 and anti-CD28, so that the nature of the TCR signal was conserved and plastic-bound antibodies were used in both cases. An altered pattern of protein phosphorylation has also been reported in response to anergy-inducing APL. Specifically, partial phosphorylation of the ζ -chains of the TCR complex was noted (Sloan-Lancaster *et al.* 1994a; Madrenas *et al.* 1995).

The second set of findings have focused on the family of small GTPases, p21^{ras} in particular. A block in the activation of p21^{ras} was described by Fields *et al.* (1996), due to a failure to convert the GDP- to the GTP-bound form. Given that the Ras pathway is important in activating the MAP kinase cascade, the reduction in extracellular signal-regulated kinase activity described by Mueller and colleagues (Li *et al.* 1996), is in keeping with a block in Ras activation.

The third, and largest set of observations, has been on the expression, activation and function of the AP-1 transcription factor complex. The first observation in this area was made by Kang *et al.* (1992) who reported reduced levels of the AP-1 complex in anergic T cells. Subsequent workers showed that some of the constituent proteins, c-Fos, FosB, and JunB, were present in reduced concentrations in anergic T cells (Mondino *et al.* 1996). The *in vitro* findings were then extended to the analysis of *in vivo* anergized T cells, in which reduced levels of c-Fos, reduced Jun N-terminal kinase-mediated post-translational modification of AP-1, and reduced binding of AP-1 to DNA were all noted (Sundstedt *et al.* 1996).

The final set of observations of altered signalling have described the presence of negative regulators of IL-2 gene transcription. The first of these was made by Becker *et al.* (1995) who observed that the nuclear factor, Nil-2a, was overexpressed in anergic T cells. The Nil-2a protein was known to bind to the negative regulatory element A in the IL-2 promoter. Although some Nil-2a was detectable in activated T cells, it was present at substantially higher concentrations in anergic T cells. A second negative regulator of T-cell activation was described by Nadler and colleagues (Boussiotis *et al.* 1997) in the form of activated GTP-Rap-1. Rap-1 is known to antagonize Ras function, and appears to be an important regulator of this key molecule. More recently another negative regulator has been described, p27kipl (Boussiotis *et al.* 2000). This is a cyclin-dependent kinase inhibitor, and associates with a co-activator of cJun, JAB1 resulting in defective transactivation of AP-1, and IL-2 transcription. Furthermore, in keeping with the predicted features of an 'anergy protein' p27kipl degradation is increased by CD28-mediated signals, and by IL-2 itself. One upstream mediator of p27kipl upregulation is cyclic AMP, a known negative regulator of T-cell activation. Cyclic AMP is also known to catalyse GTP exchange on Rap-1, raising the possibility that GTP-Rap-1, as well as inhibiting Ras activity, may contribute to the induction of p27kipl.

There may be more congruence of many of these observations than is immediately apparent. There are clearly defects in TCR-CD3-proximal signal transduction in anergic T cells, coupled with the upregulation of p59^{l^yn}. As a consequence there is defective activation of Zap-70, Ras, MAP kinases, and AP-1. At the same time Rap-1, a negative regulator of Ras, is activated as are p27kipl and Nil2a, culminating in inhibition of IL-2 gene transcrip-

tion and cell-cycle progression. These insights have important implications in the selection of immunosuppressive drugs that are 'permissive' for the induction of peripheral T-cell tolerance.

5. EVIDENCE FOR T-CELL ANERGY *IN VIVO*

As mentioned above (§ 3(h)), for several years after the *in vitro* phenomenon of T-cell anergy was first described, the majority view was that anergy represented an *in vitro* artefact, and that anergy was merely a step on the road to death (Matzinger 1994). This would appear to be a correct interpretation of anergy in B cells, the cell type in which this phenomenon was first described. Using anti-hen egg lysosome (HEL) Ig gene-transgenic mice crossed with HEL-transgenic mice, the life span of anergic B cells has been estimated to be a few hours, justifying the view that B-cell anergy is a preterminal state (Cyster *et al.* 1994). However, this does not appear to be the case for T cells. Numerous studies have described the persistence of anergic T cells, *in vivo*, for prolonged periods after the injection of the antigen that was responsible for inducing anergy.

The first description of *in vivo* T-cell anergy arose from the study of mice transgenic for an H2-E alloantigen under the rat insulin promoter (Burkly *et al.* 1989). The T cells from these mice were hyporesponsive to the transgene-encoded H2-E when challenged *in vitro*. This study raises several questions. First, how did H2-E expressed on pancreatic β -cells, in the absence of H2-M and invariant chain (Ii) expression, induce tolerance to H2-E expressed on cells replete with all the elements of the MHC class II antigen processing pathway? Data from transfection studies have indicated that alloreactive T cells raised against human class II molecules expressed in the absence of human leucocyte antigen (HLA)-DM and Ii showed little cross-reactivity with the same class II alloantigen coexpressed with these molecules (Lightstone *et al.* 1997). This is likely to be due to altered peptide occupancy. Second, in answer to one of these questions the naive T cells from the H2-E-transgenic mice would have had limited access to the ectopic H2-E molecule due to their inability to traffic through tissues. Although true for T cells in adult mice, the recent data from Arnold and colleagues (Alferink *et al.* 1998) raise the possibility that T cells may enter tissues during the neonatal period. This may allow the development of anergic or regulatory cells, specific for self-antigens, that persist and inhibit later thymic emigrants. An alternative possibility is that the transgene-encoded H2-E molecule was expressed in the thymus and induced central tolerance, as has been noted for other transgenes linked to the rat insulin promoter (Arnold & Hämmerling 1991).

Arnold and colleagues have made extensive use of transgenic mice in which the K^b alloantigen is targeted to a variety of peripheral sites using tissue-specific promoters. Reactivity to K^b in these mice was always inhibited; the mechanism of the hyporesponsiveness was determined by the site and level of expression of the K^b molecule. In some cases TCR or CD8 expression was downregulated, in others anergy appeared to be the mechanism (Schonrich *et al.* 1991, 1992). In recent studies, the issue of T-cell trafficking was investigated in this system, as referred to above (§ 5). The conclusion was that in neonatal mice,

naive T cells have access to peripheral tissues, leading to exposure to a wide range of tissue-specific autoantigens. In contrast, if naive K^b-specific T cells were introduced into adult animals they remained 'ignorant' of the allo-antigen, presumably due to lack of any exposure to the K^b molecules expressed in tissues (Alferink *et al.* 1999).

Many of the *in vivo* studies have involved the injection of bacterial or minor lymphocyte-stimulating antigen (ML) (subsequently shown to be murine mammary tumour virus proteins) SAg, which enable T cells expressing particular TCR V β families to be examined for function without further *in vitro* manipulation (Rammensee *et al.* 1989; Kawabe & Ochi 1990; Rellahan *et al.* 1991). In most of these experiments, SAg injection led to a wave of T-cell death, followed by the persistence of T cells expressing the relevant TCR V β segment but unable to respond to antigenic challenge *in vitro*, resembling the results obtained with anti-CD3 antibody treatment *in vitro*. One feature of the anergy induced *in vivo* that differs from that induced *in vitro* was that the anergic T cells were unresponsive to exogenous IL-2, even if they expressed IL-2R.

The advent of TCR-transgenic mice has enabled similar questions to be addressed using conventional antigen. Two studies have examined CD8⁺ TCR-transgenic T cells; the first made use of mice expressing a receptor specific for a lymphocytic choriomeningitis virus (LCMV) glycoprotein (Kyburz *et al.* 1993). Infection of these mice with LCMV led to a vigorous immune response, and the persistence of memory CD8⁺ T cells. In contrast, injection of the LCMV peptide for which the T cells were specific led to partial deletion of the transgenic T cells, and the induction of anergy in the remainder. The second experimental system used TCR-transgenic T cells specific for the male minor histocompatibility antigen, H-Y (Rocha & Von Boehmer 1991). H-Y-specific T cells were adoptively transferred into syngeneic mice with different numbers of male cells. The outcome was determined by the dose of antigen-bearing male cells; low numbers of cells induced priming of the transgenic T cells, intermediate numbers led to deletion, and high numbers led to a combination of deletion and anergy such that a cohort of the TCR-transgenic T cells survived but were unresponsive when rechallenged *in vitro* (Rocha *et al.* 1995). Subsequent experiments were performed with CD4⁺ TCR-transgenic T cells. Lanoue *et al.* (1997) studied mice transgenic for a TCR with specificity for a peptide of influenza haemagglutinin. It proved to be very difficult to induce unresponsiveness in the transgenic T cells, *in situ*, in the transgenic mouse. This was only achieved after partial T-cell depletion and repeated injections of antigen. However, in double transgenic mice, generated by crossing the TCR transgenics with mice transgenic for the influenza haemagglutinin, the T cells that escaped deletion in the thymus were anergic. Similarly, TCR-transgenic T cells that were adoptively transferred into haemagglutinin-transgenic hosts went through an initial phase of expansion, followed by a wave of deletion, and again the cells that survived were unresponsive. Subsequent studies from this group have observed that the 'anergic' T cells from the double transgenic mice secreted large amounts of the regulatory cytokine, IL-10, upon antigen rechallenge, raising questions concerning the mechanisms of unresponsiveness in this system (Buer *et al.* 1998).

The approach that has been pioneered by Jenkins involves the adoptive transfer of limited numbers of TCR-transgenic CD4⁺ T cells into normal syngeneic mice. This allows the transgenic T cells to function in a polyclonal environment, and for them to be analysed using clonotypic mAbs, or by labelling the cells before transfer with a fluorescent dye (Kearney *et al.* 1994). Using this model system it is clear that T-cell anergy is induced following intravenous injection of antigen (Pape *et al.* 1998). No evidence for the presence of conventional regulatory cells was obtained in this model.

Several of these *in vivo* systems have been used to address the life span of anergic T cells. In all the cases examined the anergic T cells persisted for a minimum of one month (Alferink *et al.* 1995; Pape *et al.* 1998). In the Jenkins adoptive transfer model, recovery of reactivity was observed with time, but the unresponsiveness was maintained if the antigen was injected at weekly intervals. This is reminiscent of observations made in many of the rodent transplantation tolerance models, in which anti-donor reactivity recovered slowly if the transplant was removed.

6. DOES THE INDUCTION OF T-CELL ANERGY HAVE BIOLOGICAL SIGNIFICANCE?

The context in which the induction of anergy might be thought of as having a role in the normal physiology of the immune system is in the induction and maintenance of self-tolerance. Although T-cell anergy can be induced *in vivo* by the injection of SAg, this is unlikely to relate to the induction of self-tolerance. The hypothesis that we have promoted is that autoantigen presentation by tissue parenchymal cells is a key event in the regulation of autoreactive T cells. This hypothesis is predicated on all the observations of anergy induction by co-stimulation-deficient APCs. Most of these experiments have used transfected immortalized cells as APCs with or without co-stimulatory blockade (Boussiotis *et al.* 1993). We have examined the consequences of alloantigen presentation by primary cultures of human thyroid and renal tubular epithelial cells treated with IFN- γ to induce HLA class II expression (Marelli-Berg *et al.* 1997; Frasca *et al.* 1998). The epithelial cells from both sources had a similar phenotype, most importantly they lacked detectable expression of B7 family molecules. Consistent with this phenotype they were unable to induce proliferation by HLA-DR-mismatched resting CD4⁺ T cells. This was true for both the CD45 RA (naive) and the RO (memory) subset of peripheral-blood T cells. If the T cells were cultured with the epithelial cells for five days, harvested and rested for two days, and then rechallenged with B7-expressing cells displaying the same or third-party DR molecules allospecific hyporesponsiveness was observed. Hyporesponsiveness to alloantigen in the CD45 RO cells was accompanied by increased reactivity to exogenous IL-2, indicating cell viability. The final observation made in this system was that the hyporesponsive state could be reversed by culturing the T cells in high concentrations of IL-2 after the culture with epithelial cells, and then resting them. This is strongly suggestive that anergy was the mechanism responsible for the hyporesponsive state induced by alloantigen encounter on co-stimulation-deficient parenchymal cells.

Most recently we have exploited clinical transplantation as a model system to explore these events *in vivo*. We have consistently observed the development of donor-specific hyporesponsiveness in renal and cardiac transplant patients, using limiting-dilution analysis (LDA) for cytokine-secreting CD4⁺ T cell as a read out (Mason *et al.* 1996; Hornick *et al.* 1998). If this was attributable to the *in vivo* equivalent of the observations that we had made with renal epithelial cells *in vitro* we predicted that the induction of hyporesponsiveness would be more pronounced in the CD45 RO T-cell subset. This prediction was based on the trafficking patterns of naive and memory T cells; naive T cells recirculate between blood and lymphoid tissue, while memory T cells traffic through peripheral tissues, particularly at sites of inflammation. Therefore, if encounter with the graft parenchymal cells was responsible for the induced hyporesponsiveness this should be more marked in the CD45 RO T cells. A cohort of renal transplant patients were studied immediately before, and four months after transplantation. When the ratio of pre- and post-transplant anti-donor and anti-third-party T cell frequencies were compared, a significant reduction in the anti-donor frequency was seen in the CD45 RO but not the CD45 RA T cells (R. Baker, M. Hernandez-Fuentes and R. Lechler, unpublished data). This finding is all the more striking when the fate of donor-reactive CD45 RA T cells is considered. The naive cells would have encountered emigrant donor specialized APCs in the lymph nodes draining the transplant, and converted to the RO phenotype, thereby depleting the anti-donor CD45 RA population. The final question that we addressed concerning this donor-specific hyporesponsiveness was whether it was due to deletion of anti-donor T cells, or to the induction of anergy. We exploited the fact that T-cell anergy can be reversed in *in vitro* cultured T cells; after purifying the CD4⁺ T cells from patients, some were immediately used for an LDA assay, the others were cultured for three days in exogenous IL-2 and then rested for one day, before being used in LDA. In five out of nine hyporesponsive patients a highly significant increase in frequency was seen following culture in IL-2. No change in the anti-third-party frequency was seen (F. Ng and R. Lechler, unpublished data).

Taking these data together, they support the following model concerning the *in vivo* significance of T-cell anergy induction. The peripheral T-cell repertoire contains auto-reactive potential, due to the incomplete nature of thymic deletion and the expression of tissue-sequestered and developmental antigens. When a tissue becomes inflamed, due to a tissue-tropic virus for example, the tissue-resident DCs migrate from the tissue to the draining lymph node, taking with them both the viral antigens and a large number of tissue antigens that were simultaneously internalized. In the lymph node, both virus-specific and auto-reactive T cells will be primed. The activated T cells will then traffic back to the inflamed tissue due to chemokine release and the inflamed endothelium in the tissue vasculature. Once in the tissue the virus-specific T cells will predominantly meet their peptide antigens on immigrant professional APCs that are the most efficient at capturing, processing and presenting exogenous antigen. The auto-reactive T cells, in contrast, will predominantly meet their cognate ligands on the mass of parenchymal cells that are

by this time expressing MHC class II molecules due to locally produced IFN- γ . The parenchymal cells are the cells making the autoantigen, and are thus ideally placed to process and present it. The parenchymal cell auto-antigen presentation will serve to induce anergy in the potentially dangerous T cells, as appeared to be occurring in the T cells with direct anti-donor allospecificity in the transplant patients. This model is illustrated in figure 2.

7. ANERGIC T CELLS AS PART OF THE SPECTRUM OF REGULATORY T CELLS

The above model (see §6) may partially address the biological significance of anergic T cells, but it does not provide a reason for keeping the partially activated, potentially dangerous T cell alive. Is there any advantage in maintaining a cohort of unresponsive, apparently inert cells in the system? In the final section of this review we would like to review the evidence that cells with an anergic phenotype have regulatory effects, and that this may justify their existence.

Probably the first illustration of the potential for anergic T cells to act as suppressor cells came from an SAg *in vivo* model in which MLs-1^b mice were injected with MLs-1^a cells. The Lyt-1⁺ cells (probably CD4⁺) acted as efficient suppressor cells that were antigen-non-specific in their effector function (Kawabe & Ochi 1990; Rellahan *et al.* 1991). More recently we explored the regulatory effects of anergic T cells using clonal populations of human T cells *in vitro* (Lombardi *et al.* 1994b). These cells acted as potent suppressor cells in a dose-dependent and antigen-specific manner. In addition, they were able to effect linked suppression if the APC expressed the antigen for which the anergic and the responsive T cells were specific (Frasca *et al.* 1997). Most significantly, the suppression could not be observed when anergic T cells were separated from APC and responder T cells in a transwell system, and could not be reversed by the addition of antibodies against the regulatory cytokines, IL-4, IL-10 and transforming growth factor- β . These results distinguished this phenomenon from all the other examples of T-cell-mediated regulation which were effected by soluble factors. At the time we interpreted these findings in terms of the passive 'civil servant' model of suppression first proposed by Waldmann *et al.* (1992). Subsequently we reproduced the same results in a murine system, and went on to show that the anergic T cells could prolong skin allograft survival *in vivo* (Chai *et al.* 1999), providing the first evidence that anergic T cells were capable of effecting regulation *in vivo*.

In our most recent studies (Vendetti *et al.* 2000) we have attempted to address the mechanisms whereby anergic T cells mediate their effects. Having observed that they failed to inhibit T-cell responses to combined anti-CD3 and anti-CD28 antibody stimulation, in the absence of APCs, we concluded that APCs were required for the regulatory effects to be seen. To address directly the effect of anergic T cells on APCs, bone marrow-derived DCs were generated in granulocyte monocyte-colony stimulating factor and incubated with responsive or anergic T cells specific for DC-displayed antigens. After culture with responsive T cells the immunogenicity of the DCs was increased. In marked contrast, culture with the anergic T cells rendered the DCs almost totally incapable

Figure 2. Regulatory role of induced MHC class II expression during a local immune response. The possible significance of antigen presentation by tissue parenchymal cells is illustrated. T cells are primed in the lymph node draining an inflamed tissue, by DCs that have migrated from the tissue. Both exogenous antigen-specific and autoreactive T cells are likely to be primed in response to DC presentation of both sets of antigen. Upon re-entering the tissue, through the activated endothelium, the T cells will meet their antigen *in situ*. The exogenous antigens cells will predominantly be presented by immigrant bone marrow-derived APCs that are efficient in antigen capture, processing and presentation. This will lead to reactivation of the T cells with exogenous antigen specificity. In contrast, the major cells displaying the autoantigenic epitopes will be the tissue parenchymal cells, induced to express MHC class II antigens by local IFN- γ and actively synthesizing the autoantigens. This will lead to the induction of unresponsiveness in these T cells.

of stimulating T-cell proliferation. This loss of immunogenicity was accompanied by reduced expression of MHC class II and B7 family molecules. These data suggest that inhibition of DC maturation and function may be one of the ways in which anergic T cells exert their regulatory properties. These effects are precisely the opposite of the effects of helper T cells on immature DCs, an effect which has been referred to as 'licensing'. To maintain an alcohol-related terminology, the effect of anergic T cells on DC may be referred to as 'prohibition'.

A series of very interesting observations have been made during the last few years by two groups (Sakaguchi *et al.* 1996; Suri Payer *et al.* 1998), regarding a population of pre-existing regulatory cells in the mouse. These cells were first identified in the context of a model of autoimmune disease that arose in mice that were subjected to thymectomy at three days. When Sakaguchi *et al.* (1996) compared the three-day thymectomized with normal

mice of the same strain they noted that the manipulated animals lacked a small population of peripheral T cells that were CD4⁺, CD25⁺ and CD5^{hi}. Most importantly, when these cells were transferred from a normal to a thymectomized animal they protected the recipient animal from the development of autoimmune disease. Subsequent *in vitro* studies have revealed some more features of these cells; they do not proliferate in the absence of exogenous IL-2, they do not secrete known cytokines, they require cell-to-cell contact in order to mediate their suppressive effects, they inhibit IL-2 transcription in the responsive T cells, and they appear to be antigen-non-specific in their effector function (Thornton & Shevach 2000). There are many striking similarities between this cell population and the anergic T cells that are described above. It is tempting to speculate that this CD25⁺ population arises due to encounter with self-antigens, either presented by non-immunogenic APCs, or

before the T cell is sufficiently mature to differentiate into a conventional effector cell. Indeed, they may even be generated in the thymus (Sakaguchi 2000; Seddon & Mason 2000).

At the other end of the spectrum of regulatory cells are those that regulate through the production of inhibitory or anti-inflammatory cytokines such as IL-10 (Groux *et al.* 1997). One of the best-defined *in vivo* models involving IL-10-secreting T cells is that of Powrie (Asseman *et al.* 1999), in which CD45 RB^{lo} T cells from normal animals protect lymphopenic animals from the development of inflammatory bowel disease. Clearly IL-10 is relevant to the function of these cells because the administration of anti-IL-10 antibody reverses their protective effects. However, the same protection can be provided by the CD45 RB^{lo} cells from an IL-10 knockout donor mouse (Asseman *et al.* 1999). This indicates that mechanisms other than IL-10 are used by these cells. Based on all of the available data, we propose that a spectrum of regulatory cells exists. At one end of the spectrum are cells whose regulatory function is mediated by cytokines that regulate other T cells or APCs, at the other end of the spectrum are cells whose regulatory function requires cell to cell contact and is presumably mediated via cell-surface molecular contacts involving molecules that have yet to be defined.

We have discussed the probable role of anergic T cells in the induction and maintenance of self-tolerance. Clearly, this role may well be important in transplantation tolerance. Better definition of the mechanisms whereby anergy can be induced in T cells with both direct and indirect allospecificity is a key goal in transplantation immunity. Indeed, given the ability of anergic T cells to 'prohibit' DCs, the generation of a cohort of anergic T cells with indirect allospecificity would appear to be a attractive goal in order to silence an aspect of anti-donor immunity that has been linked with chronic transplant rejection.

In conclusion, anergic T cells are alive and well, albeit in an altered state of health. Not only may the induction of anergy play a role in silencing otherwise pro-inflammatory autoreactive T cells, but their function may extend beyond this to the regulation of other potentially destructive T cells. Once the molecules involved in anergic T-cell-mediated regulation are defined, they will be attractive targets for the design of novel and more precisely targeted immunotherapeutic agents.

REFERENCES

- Alferink, J., Schitteck, B., Schonrich, G., Hammerling, G. J. & Arnold, B. 1995 Long life span of tolerant T cells and the role of antigen in maintenance of peripheral tolerance. *Int. Immunol.* **7**, 331–336.
- Alferink, J., Tafuri, A., Vestweber, D., Hallmann, R., Hammerling, G. J. & Arnold, B. 1998 Control of neonatal tolerance to tissue antigens by peripheral T cell trafficking. *Science* **282**, 1338–1341.
- Alferink, J., Aigner, S., Reibke, R., Hämmerling, G. L. & Arnold, B. 1999 Peripheral T-cell tolerance: the contribution of permission T-cell migration into parenchymal tissues of neonate. *Immunol. Rev.* **169**, 255–261.
- Arnold, B. & Hämmerling, G. J. 1991 MHC class I-transgenic mice. *A. Rev. Immunol.* **9**, 297–322.
- Arnold, B., Schonrich, G. & Hämmerling, G. J. 1993 Multiple levels of peripheral tolerance. *Immunol. Today* **14**, 12–14.
- Asseman, C., Mauze, S., Leach, M. W., Coffman, R. L. & Powrie, F. 1999 An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *J. Exp. Med.* **190**, 995–1004.
- Azuma, M., Yssel, H., Phillips, J. H., Spits, H. & Lanier, L. L. 1993 Functional expression of B7/BB1 on activated T lymphocytes. *J. Exp. Med.* **177**, 845–850.
- Becker, J. C., Brabletz, T., Krichner, T., Conrad, C. T., Brocker, E. B. & Reisfeld, R. A. 1995 Negative transcriptional regulation in anergic T cells. *Proc. Natl Acad. Sci. USA* **92**, 2375–2378.
- Bennett, S. R., Carbone, F. R., Karamalis, F., Miller, J. F. & Heath, W. R. 1997 Induction of a CD8⁺ cytotoxic T lymphocyte response by cross-priming requires cognate CD4⁺ T cell help. *J. Exp. Med.* **186**, 65–70.
- Bertoletti, A., Sette, A., Chisari, F. V., Penna, A., Levrero, M., De Carli, M., Fiaccadori, F. & Ferrari, C. 1994 Natural variants of cytotoxic epitopes are T-cell receptor antagonists for antiviral cytotoxic T cells. *Nature* **369**, 407–410.
- Beverly, B., Kang, S. M., Lenardo, M. J. & Schwartz, R. H. 1992 Reversal of *in vitro* T cell clonal anergy by IL-2 stimulation. *Int. Immunol.* **4**, 661–671.
- Bhandoola, A., Cho, E. A., Yui, K., Saragovi, H. U., Green, M. I. & Quill, H. 1993 Reduced CD3-mediated protein tyrosine phosphorylation in anergic CD4⁺ and CD8⁺ T cells. *J. Immunol.* **151**, 2355–2367.
- Boussiotis, V. A., Freeman, G. J., Gray, G., Gribben, J. & Nadler, L. M. 1993 B7 but not intercellular adhesion molecule-1 prevents the induction of human alloantigen-specific tolerance. *J. Exp. Med.* **178**, 1753–1763.
- Boussiotis, V. A., Freeman, G. J., Berezovskaya, A., Barber, D. L. & Nadler, L. M. 1997 Maintenance of human T cell anergy: blocking of IL-2 gene transcription by activated Rap1. *Science* **278**, 124–128.
- Boussiotis, V. A., Freeman, G. J., Taylor, P. A., Berezovskaya, A., Grass, I., Barber, D. L. & Nadler, L. M. 2000 P27^{kip1} functions as an anergy factor inhibiting interleukin 2 transcription and clonal expansion of alloreactive human and mouse helper T lymphocytes. *Nature Med.* **6**, 290–297.
- Bretscher, P. & Cohn, M. 1970 A theory of self–nonself discrimination. *Science* **169**, 1042–1049.
- Buer, J., Lanoue, A., Franzke, A., Garcia, C., Von Boehmer, H. & Sarukhan, A. 1998 Interleukin-10 secretion and impaired effector function of major histocompatibility complex class II-restricted T cells anergized *in vivo*. *J. Exp. Med.* **187**, 177–183.
- Burkly, L. C., Lo, D., Kanagawa, O., Brinster, R. L. & Flavell, R. A. 1989 T cell tolerance by clonal anergy in transgenic mice with nonlymphoid expression of MHC class II I-E. *Nature* **342**, 564–566.
- Cardin, R. D., Brooks, J. W., Sarawar, S. R. & Doherty, P. C. 1996 Progressive loss of CD8⁺ T cell-mediated control of a gamma-herpesvirus in the absence of CD4⁺ T cells. *J. Exp. Med.* **184**, 863–871.
- Chai, J. G. & Lechler, R. I. 1997 Immobilized anti-CD3 mAb induces anergy in murine naive and memory CD4⁺ T cells. *Int. Immunol.* **9**, 935–944.
- Chai, J. G., Bartok, I., Scott, D., Dyson, J. & Lechler, R. 1998 T/T antigen presentation by activated murine CD8⁺ T cells induces anergy and apoptosis. *J. Immunol.* **160**, 3655–3665.
- Chai, J. G., Bartok, I., Chandler, P., Vendetti, S., Antoniou, A., Dyson, J. & Lechler, R. 1999 Anergic T cells act as suppressor cells *in vitro* and *in vivo*. *Eur. J. Immunol.* **29**, 686–692.
- Cho, E. A., Riley, M. P., Sillman, A. L. & Quill, H. 1993 Altered protein tyrosine phosphorylation in anergic Th1 cells. *J. Immunol.* **151**, 20–28.
- Cyster, J. G., Hartley, S. B. & Goodnow, C. C. 1994 Competition for follicular niches excludes self-reactive B cells from the recirculating B-cell repertoire. *Nature* **371**, 389–395.

- Davis, L. S. & Lipsky, P. E. 1993 Tolerance induction of human CD4⁺ T cells: markedly enhanced sensitivity of memory versus naive T cells to peripheral anergy. *Cell. Immunol.* **146**, 351–361.
- Deeths, M. J., Kedl, R. M. & Mescher, M. F. 1999 CD8⁺ T cells become nonresponsive (anergic) following activation in the presence of costimulation. *J. Immunol.* **163**, 102–110.
- De Magistris, M. T., Alexander, J., Coggeshall, M., Altman, A., Gaeta, F. C., Grey, H. M. & Sette, A. 1992 Antigen analog-major histocompatibility complexes act as antagonists of the T cell receptor. *Cell* **68**, 625–634.
- DeSilva, D. R., Urdahl, K. B. & Jenkins, M. K. 1991 Clonal anergy is induced by T cell receptor occupancy in the absence of proliferation. *J. Immunol.* **147**, 3261–3267.
- Fields, P. E., Gajewski, T. F. & Fitch, F. W. 1996 Blocked Ras activation in anergic CD4⁺ T cells. *Science* **271**, 1276–1278.
- Frasca, L., Carmichael, P., Lechler, R. & Lombardi, G. 1997 Anergic T cells effect linked suppression. *Eur. J. Immunol.* **27**, 3191–3197.
- Frasca, L., Marelli-Berg, F., Imami, N., Potolicchio, I., Carmichael, P., Lombardi, G. & Lechler, R. I. 1998 Gamma-interferon treated renal tubular epithelial cells induce allo-specific tolerance. *Kidney Int.* **53**, 679–689.
- Frasca, L., Tamir, A., Jurcevic, S., Marinari, B., Monizio, A., Sorrentino, R., Carbonari, M., Piccolella, E., Lechler, R. I. & Lombardi, G. 2000 Peptide analogues as a strategy to induce tolerance in T cells with indirect allospecificity. *Transplantation* **70**, 1–10.
- Frauwirth, K. A., Alegre, M. L. & Thompson, C. B. 2000 Induction of T cell anergy in the absence of CTLA-4/B7 interaction. *J. Immunol.* **164**, 2987–2993.
- Freeman, G. J., Freeman, A. S., Segil, J. M., Lee, G., Whiteman, J. F. & Nadler, L. M. 1989 B7, a new member of the Ig superfamily with unique expression on activated and neoplastic B cells. *J. Immunol.* **143**, 2714–2722.
- Gajewski, T. F., Qian, D., Field, P. & Fitch, F. W. 1994 Anergic T-lymphocyte clones have altered inositol phosphate, calcium and tyrosine kinase signalling pathways. *Proc. Natl Acad. Sci. USA* **91**, 38–42.
- Groux, H., Bigler, M., de Vries, J. E. & Roncarolo, M.-G. 1996 Interleukin-10 induces a long-term antigen-specific anergic state in human CD4⁺ T cells. *J. Exp. Med.* **184**, 19–29.
- Groux, H., O'Garra, A., Bigler, M., Rouleau, M., Antonenko, S., de Vries, J. E. & Roncarolo, M.-G. 1997 A CD4⁺ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* **389**, 737–742.
- Groux, H., Bigler, M., de Vries, J. E. & Roncarolo, M. G. 1998 Inhibitory and stimulatory effects of IL-10 on human CD8⁺ T cells. *J. Immunol.* **160**, 3188–3193.
- Guinan, E. C., Bousiotis, V. A., Neuberger, D., Brennan, L. L., Hirano, N., Nadler, L. M. & Gribben, J. G. 1999 Transplantation of anergic histoincompatible bone marrow allografts. *New Engl. J. Med.* **340**, 1704–1714.
- Hargreaves, R. G., Borthwick, N. J., Montani, M. S. G., Piccolella, E., Carmichael, P., Lechler, R. I., Akbar, A. N. & Lombardi, G. 1997 Dissociation of T cell anergy from apoptosis by blockade of Fas/Apo-1 (CD95) signaling. *J. Immunol.* **158**, 3099–3107.
- Hayashi, R. J., Loh, D. Y., Kanagawa, O. & Wang, F. 1998 Differences between responses of active and activated T cells to anergy induction. *J. Immunol.* **160**, 33–38.
- Hollberg, P., Scholz, C., Anderson, D. E., Greenfield, E. A., Kuchroo, V. K., Freeman, G. J. & Hafler, D. A. 1997 Expression of a hypoglycosylated form of CD86 (B7-2) on human T cells with altered binding properties to CD28 and CTLA-4. *J. Immunol.* **159**, 4799–4805.
- Hornick, P. I., Mason, P. D., Yacoub, M. H., Rose, M. L., Batchelor, R. & Lechler, R. I. 1998 Assessment of the contribution that direct allorecognition makes to the progression of chronic cardiac transplant rejection in humans. *Circulation* **97**, 1257–1263.
- Jenkins, M. K. & Schwartz, R. H. 1987 Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness *in vitro* and *in vivo*. *J. Exp. Med.* **165**, 302–319.
- Jenkins, M. K., Pardoll, D., Mizuguchi, J., Chused, T. M. & Schwartz, R. H. 1987 Molecular events in the induction of a nonresponsive state in interleukin 2-producing helper T-lymphocyte clones. *Proc. Natl Acad. Sci. USA* **84**, 5409–5413.
- Judge, T. A., Wu, Z., Zhang, X. G., Sharpe, A. H., Sayegh, M. H. & Turka, L. A. 1999 The role of CD80, CD86 and CTLA-4 in alloimmune response and the induction of long-term allograft survival. *J. Immunol.* **162**, 1947–1951.
- Kang, S. M., Beverly, B., Tran, A. C., Brorson, K., Schwartz, R. H. & Lenardo, M. L. 1992 Transactivation by AP-1 is a molecular target of T cell clonal anergy. *Science* **257**, 1134–1138.
- Kawabe, Y. & Ochi, A. 1990 Selective anergy of $\nu\beta 8^{+}$ CD4⁺ T cells in staphylococcus enterotoxin B-primed mice. *J. Exp. Med.* **172**, 1065–1070.
- Kearney, E. R., Pape, K. A., Loh, D. Y. & Jenkins, M. K. 1994 Visualization of peptide-specific T cell immunity and peripheral tolerance induction *in vivo*. *Immunity* **1**, 327–339.
- Kirberg, J., Bruno, L. & Von Boehmer, H. 1993 CD4⁺ help prevents rapid deletion of CD8⁺ cells after a transient response to antigen. *Eur. J. Immunol.* **23**, 1963–1967.
- Klenerman, P. (and 12 others) 1994 Cytotoxic T-cell activity antagonized by naturally occurring HIV-1 Gag variants. *Nature* **369**, 403–407.
- Krummel, M. F. & Allison, J. P. 1995 CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. *J. Exp. Med.* **182**, 459–465.
- Kyburz, D., Aichele, P., Speiser, D. E., Hengartner, H., Zinknagel, R. M. & Pircher, H. 1993 T cell immunity after a viral infection versus T cell tolerance induced by soluble viral peptides. *Eur. J. Immunol.* **23**, 1956–1962.
- Lafferty, K. J. & Gill, R. G. 1993 The maintenance of self-tolerance. *Immunol. Cell. Biol.* **71**, 209–214.
- Lamb, J. R. & Feldmann, M. 1984 Essential requirement for major histocompatibility complex in recognition in T cell tolerance induction. *Nature* **308**, 72–74.
- Lamb, J. R., Skidmore, B. J., Green, N., Chiller, J. M. & Feldmann, M. 1983 Induction of tolerance in influenza virus-immune T lymphocyte clones with synthetic peptides of influenza hemagglutinin. *J. Exp. Med.* **157**, 1434–1447.
- Lanoue, A., Bona, C., Von Boehmer, H. & Sarukhan, A. 1997 Conditions that induce tolerance in mature CD4⁺ T cells. *J. Exp. Med.* **185**, 405–414.
- Lee, K. M., Chuang, E., Griffin, M., Khattri, R., Hong, D. K., Zhang, W., Straus, D., Samelson, L. E., Thompson, C. B. & Bluestone, J. A. 1998 Molecular basis of T cell inactivation by CTLA-4. *Science* **282**, 2263–2266.
- Li, W., Whaley, C. D., Mondino, A. & Mueller, D. L. 1996 Blocked signal transduction to the ERK and JNK protein kinases in anergic CD4⁺ T cells. *Science* **271**, 1272–1275.
- Lightstone, L., Hargreaves, R., Bobek, G., Peterson, M., Aichinger, G., Lombardi G. & Lechler, R. 1997 In the absence of the invariant chain, HLA-DR molecules display a distinct array of peptides which is influenced by the presence or absence of HLA-DM. *Proc. Natl Acad. Sci. USA* **94**, 5772–5777.
- Linsley, P. S., Brady, W., Urnes, M., Grosmaire, L. S., Damle, N. K. & Ledbetter, J. A. 1991a Binding of the B cell activation antigen B7 to CD28 costimulate T cell proliferation and interleukin-2 mRNA accumulation. *J. Exp. Med.* **173**, 721–730.
- Linsley, P. S., Brady, W., Urnes, M., Grosmaire, L. S., Damle, N. K. & Ledbetter, J. A. 1991b CTLA-4 is a second receptor for the B-cell activation antigen B7. *J. Exp. Med.* **174**, 561–569.

- Lloyd, T. E., Yang, L., Tang, D. N., Bennett, T., Schober, W. & Lewis, D. E. 1997 Regulation of CD28 costimulation in human CD8⁺ T cells. *J. Immunol.* **158**, 1551–1558.
- Lombardi, G., Sidhu, S., Dodi, T., Batchelor, R. & Lechler, R. 1994a Failure of correlation between B7 expression and activation of interleukin-2-secreting T cells. *Eur. J. Immunol.* **24**, 523–530.
- Lombardi, G., Sidhu, S., Batchelor, R. & Lechler, R. I. 1994b Anergic T cells act as suppressor cells *in vitro*. *Science* **264**, 1587–1589.
- Lombardi, G., Hargreaves, R., Sidhu, S., Imami, N., Lightstone, L., Fuller-Espie, S., Ritter, M., Robinson, P., Tarnok, A. & Lechler, R. 1996 Antigen presentation by T cells inhibits IL-2 production and induces IL-4 release due to altered cognate signals. *J. Immunol.* **156**, 2769–2775.
- Lord, G. M., Lechler, R. I. & George, A. J. 1999 A kinetic differentiation model for the action of altered TCR ligands. *Immunol. Today* **20**, 33–39.
- Lyons, D. S., Lieberman, S. A., Hampl, J., Boniface, J. J., Chien, Y., Berg, L. J. & Davis, M. M. 1996 A TCR binds to antagonist ligands with lower affinities and faster dissociation rates than to agonists. *Immunity* **5**, 53–61.
- Madrenas, J., Wange, R. L., Wang, J. L., Isakov, N., Samelson, L. E. & Germain, R. N. 1995 Zeta phosphorylation without ZAP-70 activation induced by TCR antagonists or partial agonists. *Science* **267**, 515–518.
- Marelli-Berg, F., Weetman, A., Frasca, F., Deacock, S., Imami, N., Lombardi, G. & Lechler, R. I. 1997 Antigen presentation by epithelial cells induces anergic immunoregulatory CD45RO⁺ T cells and deletion of CD45RA cells. *J. Immunol.* **159**, 5853–5861.
- Mason, P. D., Robinson, C. M. & Lechler, R. I. 1996 Detection of donor-specific hyporesponsiveness following late failure of human renal allografts. *Kidney Int.* **50**, 1019–1025.
- Matloubian, M., Concepcion, R. J. & Ahmed, R. 1994 CD4⁺ T cells are required to sustain CD8⁺ cytotoxic T-cell responses during chronic viral infection. *J. Virol.* **68**, 8056–8063.
- Matzinger, P. 1994 Tolerance, danger and the extended family. *A. Rev. Immunol.* **12**, 991–1045.
- Mondino, A., Whaley, C. D., DeSilva, D. R., Li, W., Jenkins, M. K. & Mueller, D. L. 1996 Defective transcription of the IL-2 gene is associated with impaired expression of c-Fos, Fos-B and JunB in anergic T helper 1 cells. *J. Immunol.* **157**, 2048–2057.
- Mueller, D. L., Chiodetti, L., Bacon, P. L. & Schwartz, R. N. 1991 Clonal anergy blocks the response to IL-4, as well as the production of IL-2 in dual-producing helper T cell clones. *J. Immunol.* **147**, 4118–4125.
- Nossal, G. J. & Pick, B. L. 1980 Clonal anergy: persistence in tolerant mice of antigen-binding B lymphocytes incapable of responding to antigen or mitogen. *Proc. Natl Acad. Sci. USA* **77**, 1602–1606.
- Ossendorp, F., Mengede, E., Camps, M., Filius, R. & Melief, C. J. 1998 Specific T helper cell requirement for optimal induction of cytotoxic T lymphocytes against major histocompatibility complex class II negative tumors. *J. Exp. Med.* **187**, 693–702.
- Otten, G. R. & Germain, R. N. 1991 Split anergy in a CD8⁺ T cell receptor-dependent cytotoxicity in the absence of interleukin-2 production. *Science* **251**, 1228–1231.
- Pape, K. A., Merica, R., Mondino, A., Khoruts, A. & Jenkins, M. K. 1998 Direct evidence that functionally impaired CD4⁺ T cells persist *in vivo* following induction of peripheral tolerance. *J. Immunol.* **160**, 4719–4729.
- Perez, V. L., Parijs, L. V., Biuckians, A., Zhang, X. X., Strom, T. B. & Abbas, A. K. 1997 Induction of peripheral T cell tolerance *in vivo* requires CTLA-4 engagement. *Immunity* **6**, 411–417.
- Powell, J. D., Lerner, C. G. & Schwartz, R. H. 1999 Inhibition of cell cycle progression by rapamycin induces T cell clonal anergy even in the presence of costimulation. *J. Immunol.* **162**, 2775–2784.
- Quill, H. A. & Schwartz, R. H. 1987 Stimulation of normal inducer T cell clones with antigen presented by purified Ia molecules in planar lipid membranes: specific induction of a long-lived state of proliferative non-responsiveness. *J. Immunol.* **138**, 3704–3709.
- Quill, H., Riley, M. P., Cho, E. A., Casnellie, J. E., Reed, J. C. & Torigoe, T. 1992 Anergic Th1 cells express altered levels of the protein tyrosine kinases p56lck and p59fyn. *J. Immunol.* **149**, 2887–2893.
- Racioppi, L., Ronchese, F., Matis, L. A. & Germain, R. N. 1993 Peptide-major histocompatibility complex class II complexes with mixed agonist/antagonist properties provide evidence for ligand-related differences in T cell receptor-dependent intracellular signaling. *J. Exp. Med.* **177**, 1047–1060.
- Rammensee, H. G., Kroschewski, R. & Frangoulis, B. 1989 Clonal anergy induced in mature V β 6⁺ T lymphocytes on immunizing Mls-1b mice with Mls-1a expressing cells. *Nature* **339**, 541–544.
- Rellahan, B. L., Jones, L. A., Kruisbeek, A. M., Fry, A. M. & Matis, L. A. 1991 *In vivo* induction of anergy in peripheral V β 8⁺ T cells by staphylococcal enterotoxin B. *J. Exp. Med.* **172**, 1091–1100.
- Rocha, B. & Von Boehmer, H. 1991 Peripheral selection of the T cell repertoire. *Science* **251**, 1225–1228.
- Rocha, B., Grandien, A. & Freitas, A. A. 1995 Anergy and exhaustion are independent mechanisms of peripheral T cell tolerance. *J. Exp. Med.* **181**, 993–984.
- Sagerstorm, C. G., Kerr, E. M., Allison, J. P. & Davis, M. M. 1993 Activation and differentiation requirements of primary T cells *in vitro*. *Proc. Natl Acad. Sci. USA* **90**, 8987–8991.
- Sakaguchi, S. 2000 Regulatory T cells: key controllers of immunologic self-tolerance. *Cell* **101**, 455–458.
- Sakaguchi, S., Toda, M., Asano, M., Itoh, M., Morse, S. S. & Sakaguchi, N. 1996 T cell-mediated maintenance of natural self-tolerance: its breakdown as possible cause of various autoimmune diseases. *J. Autoimmun.* **9**, 211–220.
- Sansom, D. M. & Hall, N. D. 1993 B7/BB1, the ligand for CD28, is expressed on repeatedly activated human T cells *in vitro*. *Eur. J. Immunol.* **23**, 295–298.
- Schonrich, G., Kalinke, U., Momburg, F., Malissen, M., Schmitt-Verhulst, A. M., Malissen, B., Hammerling, G. J. & Arnold, B. 1991 Down-regulation of T cell receptors on self-reactive T cells as a novel mechanism for extrathymic tolerance induction. *Cell* **65**, 293–304.
- Schonrich, G., Momburg, F., Malissen, M., Schmitt-Verhulst, A. M., Malissen, B., Hammerling, G. J. & Arnold, B. 1992 Distinct mechanisms of extrathymic T cell tolerance due to differential expression of self-antigen. *Int. Immunol.* **4**, 581–590.
- Schwartz, R. H. 1996 Models of T cell anergy: is there a common molecular mechanism? *J. Exp. Med.* **184**, 1–8.
- Seddon, B. & Mason, D. 2000 The third function of the thymus. *Immunol. Today* **21**, 95–99.
- Sidhu, S., Deacock, S., Bal, V., Batchelor, R., Lombardi, G. & Lechler, R. 1992 Human T cells cannot act as autonomous antigen presenting cells but induce tolerance in antigen-specific and alloreactive T cells. *J. Exp. Med.* **176**, 875–880.
- Sloan-Lancaster, J., Evavold, B. D. & Allen, P. M. 1993 Induction of T-cell anergy by altered T-cell-receptor ligand on live antigen-presenting cells. *Nature* **363**, 156–159.
- Sloan-Lancaster, J., Shaw, A. S., Rothbard, J. B. & Allen, P. M. 1994a Partial T cell signalling: altered phospho-zeta and lack of Zap-70 recruitment in APL-induced T cell anergy. *Cell* **79**, 913–922.

- Sloan-Lancaster, J., Evavold, B. D. & Allen, P. M. 1994b Th2 cell clonal anergy as a consequence of partial activation. *J. Exp. Med.* **180**, 1195–1205.
- Steinbrink, K., Jonuleit, H., Muller, G., Schuler, G., Knop, J. & Enk, A. H. 1999 Interleukin-10-treated human dendritic cells induce a melanoma-antigen-specific anergy in CD8(+) T cells resulting in a failure to lyse tumor cells. *Blood* **93**, 1634–1642.
- Sundstedt, A., Sigvardsson, M., Leanderson, T., Hedlund, G., Kalland, T. & Dohlsten, M. 1996 *In vivo* anergized CD4⁺ T cells express perturbed AP-1 and NF- κ B transcription factors. *Proc. Natl Acad. Sci. USA* **93**, 979–984.
- Suri-Payer, E., Amar, A. Z., Thornton, A. M. & Shevach, E. M. 1998 CD4⁺CD25⁺ T cells inhibit both the induction and effector function of autoreactive T cells and represent a unique lineage of immunoregulatory cells. *J. Immunol.* **160**, 1212–1218.
- Thornton, A. M. & Shevach, E. M. 2000 Suppressor effector function of CD4⁺CD25⁺ immunoregulatory T cells is antigen nonspecific. *J. Immunol.* **164**, 183–190.
- Tivol, E. A., Borriello, F., Schweitzer, A. N., Lynch, W. P., Bluestone, J. A. & Sharpe, A. H. 1995 Loss of CTLA-4 leads to massive lymphoproliferative and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity* **3**, 541–547.
- Tsitoura, D. C., DeKruyff, R. H., Lamb, J. R. & Umetsu, D. T. 1999 Intranasal exposure to protein antigen induces immunological tolerance mediated by functionally disabled CD4⁺ T cells. *J. Immunol.* **163**, 2592–2600.
- Vendetti, S., Chai, J. G., Dyson, J., Simpson, E., Lombardi, G. & Lechler, R. 2000 Anergic T cells inhibit the antigen-presenting function of dendritic cells. *J. Immunol.* **165**, 1175–1181.
- Waldmann, H., Qin, S. & Cobbold, S. 1992 Monoclonal antibodies as agents to reinduce tolerance in autoimmunity. *J. Autoimmun.* **5**, 93–102.
- Walunas, T. L. & Bluestone, J. A. 1998 CTLA-4 regulates tolerance induction and T cell differentiation *in vivo*. *J. Immunol.* **160**, 3855–3860.
- Walunas, T. L., Lenschow, D. L., Bakker, C. Y., Linsley, P. S., Freeman, G. J., Green, J. M., Thompson, C. B. & Bluestone, J. A. 1994 CTLA-4 can function as a negative regulator of T cell activation. *Immunity* **1**, 405–413.
- Waterhouse, P., Penninger, J. M., Timms, E., Wakeham, A., Shahinian, A., Lee, K. L., Thompson, C. B., Griesser, H. & Tak, M. T. 1995 Lymphoproliferative disorders with early lethality in mice deficient in CTLA-4. *Science* **270**, 985–988.
- Williams, M. E., Shea, C. M., Lichtman, A. H. & Abbas, A. K. 1992 Antigen receptor-mediated anergy in resting T lymphocytes and T cell clones. Correlation with lymphokine secretion patterns. *J. Immunol.* **149**, 1921–1926.
- Wolf, H., Muller, Y., Salmen, S., Wilmanns, W. & Jung, G. 1994 Induction of anergy in resting human T lymphocytes by immobilized anti-CD3 antibodies. *Eur. J. Immunol.* **24**, 1410–1417.
- Wyss-Coray, T., Mauri-Hellweg, D., Baumann, K., Bettens, F., Grunow, R. & Pichler, W. J. 1993 The B7 adherent molecule is expressed on activated human T cells: functional involvement in T–T interactions. *Eur. J. Immunol.* **23**, 2175–2180.
- Zheng, X. X., Markees, T. G., Hancock, W. W., Li, Y., Greiner, D. L., Li, X. C., Mordes, J. P., Sayegh, M. H., Rossini, A. A. & Strom, T. B. 1999 CTLA-4 signals are required to optimally induce allograft tolerance with combined donor-specific transfusion and anti-CD154 monoclonal antibody treatment. *J. Immunol.* **162**, 4983–4990.