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T-cell anergy

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Self-reactive T cells that escape negative selection in the thymus must be inactivated in the periphery. Anergy constitutes one means of imposing peripheral tolerance. Anergic T cells are functionally inactivated and unable to initiate a productive response even when antigen is encountered in the presence of full co-stimulation. Recent studies have provided new insights into the mechanisms responsible for the induction and maintenance of T-cell anergy. These studies have helped clarify the nature of the signals that induce tolerance, the cells able to deliver them and the molecular processes that underlie the unresponsive state.

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Current Opinion in Immunology 2004, **16**:209–216

This review comes from a themed issue on Lymphocyte development
Edited by Leslie Berg and Michel Nussenzweig

0952-7915/$ – see front matter
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DOI 10.1016/j.coi.2004.01.013

Abbreviations

CTLA-4	cytotoxic T lymphocyte antigen 4
DC	dendritic cell
GM-CSF	granulocyte-macrophage colony-stimulating factor
IFN	interferon
IL	interleukin
ILT	Ig-like transcript
NFAT	nuclear factor of activated T cells
TCR	T-cell receptor
TGF	transforming growth factor
Th	T helper
TNF	tumor necrosis factor
Tr	T regulatory

Introduction

T cells can discriminate between peptide antigens with an exquisite degree of specificity, but the T-cell receptor (TCR) is not intrinsically capable of distinguishing self from non-self. The majority of self-reactive T cells are clonally deleted in the thymus, following recognition of self-antigens expressed on thymic stromal cells [1]. T cells that have exited the thymus remain capable of making responses to self-antigens, however, and their ability to distinguish self from non-self in peripheral lymphoid tissues appears to be conferred by recognition of co-stimulatory molecules on antigen-presenting cells. Because co-stimulatory proteins are upregulated during inflammation, infection and other pathological conditions, sensing their level of expression is an ideal means of enabling T cells to make the distinction between 'non-infectious self' and 'infectious non-self' [2].

In this review we have attempted to analyze the large body of information suggesting that lack of co-stimulation leads to a state of functional unresponsiveness that has been termed 'anergy'. When co-stimulatory signals are present, T cells proliferate and proceed to make a full-fledged immune response. By contrast, when co-stimulatory signals are absent, T cells become anergic — unresponsive to secondary stimulation, even if this includes both TCR and co-stimulatory signals. Thus, co-stimulation provides not only the second signal that is needed for a T cell to proliferate, it also provides signals that prevent anergy induction.

Activating and tolerogenic signals provided by co-stimulatory proteins

Although a large number of co-stimulatory ligand–receptor pairs are now known, the CD28–CTLA-4–B7 triad remains the best characterized. The CD28 and CTLA-4 (cytotoxic T lymphocyte antigen 4) receptors on T cells both bind the ligands B7-1 (CD80) and B7-2 (CD86) on antigen-presenting cells (CTLA-4 with ~10-fold higher affinity than CD28) but exert positive and negative influences on T-cell activation, respectively (reviewed in [3–5]). Although the simple absence of co-stimulation is sufficient to induce anergy in effector T cells and T-cell clones *in vitro*, CTLA-4 engagement may be necessary to induce anergy in naïve $CD4^{+}$ T cells *in vivo*, as judged by the fact that $CTLA4^{-/-}$ cells are significantly more resistant to a tolerizing regimen that involves adoptive transfer and stimulation with soluble antigen in comparison to wild-type T cells [6].

Because CTLA-4 is expressed at high levels only after T-cell activation, one interpretation of these data is that anergy induction in naïve T cells requires the previous step of suboptimal activation. Indeed, numerous studies show that naïve T cells receiving a tolerogenic stimulus undergo an initial activation and/or expansion phase before achieving a tolerant state. During this phase they

may demonstrate effector functions similar to those demonstrated by their counterparts that have received immunogenic stimuli [7,8•]. In one study, anergy induction in of naïve $CD4^+$ T cells *ex vivo* depended on suboptimal co-stimulation mediated through binding of the T-cell integrin leukocyte function-associated antigen (LFA-1) to its ligand intercellular adhesion molecule (ICAM-1) [9]. Thus, a possible mechanism for tolerance induction in naïve cells *in vivo* is that an initial phase of activation and/or expansion is followed by high-level engagement of CTLA-4, which imposes anergy by attenuating CD28 signaling. In this scenario, the major target of anergy *in vivo* is not the naïve T cell, but rather a preactivated (or partially preactivated) T cell that might or might not have acquired some effector function.

To explain the resistance of CTLA-$4^{-/-}$ T cells to tolerance induction *in vivo*, an alternative view is that CTLA-4 directly suppresses the response of naïve T cells, which express low levels of surface CTLA-4 [5]. CTLA-4 signaling appears to prevent cell cycle entry and cause cell cycle arrest [10,11], and several *ex vivo* studies indicate that blocking cell cycle progression in naïve T cells induces anergy even in the presence of adequate co-stimulation [12,13]. The form of T-cell anergy induced by cell cycle blockade appears to differ mechanistically from that induced by co-stimulation blockade [10], thus both mechanisms may operate during tolerance induction *in vivo*. In contrast to $CD4^+$ T cells, $CD8^+$ cells can become anergic in the absence of CTLA-4 and might even require CD28 co-stimulation [14,15]. A major determinant of tolerance in $CD8^+$ T cells is lack of $CD4^+$ T-cell help [16].

In addition to CD28, several other molecules function as co-stimulatory molecules for T cells. Most belong to the extended CD28–B7 family, which includes inducible co-stimulatory molecule (ICOS)–B7h and programmed death 1 (PD1)–PD1L1/L2, whereas others belong to the TNF–TNFR family (OX40–OX40L, 4-1BB–4-1BBL, TNF-related activation-induced cytokine [TRANCE]–receptor activator of NF-κB [RANK], CD70–CD27 and CD153–CD30) [17]. Corresponding to the opposing properties of CD28 and CTLA-4, some of these molecules have positive and some have negative co-stimulatory functions; moreover, different ligand–receptor pairs might act in different cell types. Thus, ligation of CD137, a member of the TNF superfamily, prevents anergy induction in cytolytic T cells [18], whereas blockade of two other members of the TNF superfamily, Light and CD40, induces T-cell anergy and prevents graft versus host disease [19].

Dendritic cells can deliver tolerogenic signals

Maturation status determines dendritic-cell function

Dendritic cells (DCs) play key roles in T-cell activation, as they are extremely effective in priming naïve T cells. DC maturation is required for optimal antigen presentation: immature DCs are active in antigen uptake and processing but show only moderate surface expression of MHC class II and little or no expression of co-stimulatory molecules (e.g. B7, CD40). DC maturation is stimulated by lipopolysaccharide (LPS) and various cytokines (IL-1, GM-CSF, TNF-α), and the resulting mature DCs express much higher levels of co-stimulatory molecules and MHC, and are significantly more capable of eliciting T-cell activation.

Recent evidence indicates that DCs are also pivotal in regulating immune tolerance. In general, tolerogenic DCs correspond to immature DCs, which bear low levels of co-stimulatory molecules, whereas immunogenic DCs have matured to express high levels of MHC molecules as well as co-stimulatory ligands. Thus, the immunosuppressive cytokine IL-10 interferes with DC maturation, inhibiting expression of MHC class II, co-stimulatory proteins and secretion of inflammatory cytokines [20]; it also confers tolerogenic capabilities on DCs, which are then able to induce T cells with suppressor activities [21•,22•]. Tolerogenic DCs can also be generated *ex vivo* by treating them with TGF-β or a variety of immunosuppressive drugs that inhibit DC maturation (e.g. cyclosporine A and FK506, rapamycin, glucocorticoids such as dexamethasone, aspirin, vitamin D, N-acetyl-L-cysteine and deoxyspergualin [23–25]). DCs in many tumors lose their immunostimulatory functions in concert with decreased expression of co-stimulatory molecules, apparently because their maturation is prevented by immunosuppressive cytokines secreted by the tumors [26]. This process might be largely responsible for the development of immune tolerance to tumors.

Characteristics of tolerizing populations of dendritic cells

Specific subsets of immature tolerizing DCs have been identified and characterized by their function and expression of surface markers. The recurring theme is that, despite different protocols for generation and the expression of different surface phenotypes, tolerogenic DCs bear low levels of co-stimulatory molecules and often low levels of MHC proteins.

Specific subsets of immature tolerizing DCs have been identified and characterized by their function and expression of surface markers. The recurring theme is that, despite different protocols for generation and different surface phenotypes, tolerogenic DCs bear low levels of co-stimulatory molecules and often also low levels of MHC proteins. Thus, the targeting of peptides to immature DCs leads to tolerogenic presentation to both $CD4^+$ and $CD8^+$ T cells. Maturation signals provided by CD40 engagement transform these signals and the DCs turn from being tolerogenic to inducing T-cell activation and proliferation [27,28•,29]. Similarly, $CD11c^{low}CD45RB^{high}$

DCs, obtained by culturing bone marrow cells with combined IL-10, GM-CSF and TNF-α, were tolerogenic and expressed low levels of MHC class II, CD80 and CD86 [30•]. DCs with a similar surface phenotype were identified in spleen and lymph nodes of normal mice and were shown to be significantly enriched in IL-10 transgenic mice.

CD40 plays a crucial role in DC maturation and immunogenicity, as apparent from the fact that DCs from $CD40^{-/-}$ mice are tolerogenic with low-level expression of MHC class II and CD86 [31]. NK/DC ($CD11c^+/DX5^+$), bitypic regulatory cells sharing the phenotype and functional properties of NK cells and DCs, were shown to mediate tolerance induced by CD40L blockade in a model of virally induced type 1 diabetes. These cells possessed antigen-presenting cell function but expressed reduced levels of MHC class II [32]. Inhibition of CD40-mediated signaling and NF-κB activation generated $CD8^+CD28^-$ suppressor T cells, which provoked increased expression of the Ig-like inhibitory receptors Ig-like transcript 3 (ILT3), ILT4 on human immature DCs. The resulting DCs were tolerogenic, did not express CD80 or CD86, and were capable of anergizing $CD4^+$ T cells [33•].

As discussed in a later section, however, some tolerogenic properties of DCs are more attributable to their effects on T-cell differentiation, cytokine production and regulatory T-cell function than to direct induction of anergy through decreased co-stimulatory function. Yet another mechanism of tolerance induction by DCs is associated with increased expression of indoleamine 2,3 dioxygenase, a tryptophan-catabolizing enzyme [34•,35•].

Clinical applications of co-stimulatory blockade

The goal in treating autoimmune and transplant patients is to re-establish specific tolerance to self-antigen, without causing generalized immunosuppression. Many of the strategies attempted have been shown to work at least in part through co-stimulatory blockade and the resulting development of anergy. Graft survival has been prolonged by blocking CD28–B7 interactions with CTLA-4Ig, either alone or in combination with anti-CD154, which blocks CD40–CD40L interactions [36,37]. However, permanent tolerance is not achieved unless the cell cycle inhibitor rapamycin is added to these regimens [38], consistent with the hypothesis that cell cycle blockade and co-stimulatory blockade induce distinct but complementary forms of tolerance. In bone marrow transplantation, co-stimulatory blockade elicits long-lasting tolerance [37]. Host $CD4^+$ T cells that are donor reactive are first anergized, and they maintain tolerance until they are deleted in the periphery [39,40], whereas regulatory T cells seem to play a role in suppressing $CD8^+$ but not $CD4^+$ T cells [37]. In other cases, co-stimulatory blockade alone has failed to support permanent engraftment despite prolonged acceptance of a graft, probably due to the fact that $CD8^+$ T cells are less dependent on CD28–CD40 co-stimulation than $CD4^+$ T cells [41,42].

Attempts to treat autoimmune diseases have included systemic or mucosal administration of antigens or altered peptide ligands, which elicit TCR stimulation in the absence of co-stimulation. Tolerance induction depends on the dose and physical–chemical form of the antigen as well as the route of administration. Oral or intravenous administration of soluble proteinaceous antigens causes anergy or deletion [6,43]. The use of recombinant MHC peptide complexes is also useful at eliciting anergy [44]. The administration of antigens in a tolerogenic form has been attempted in autoimmune diseases in humans [45], and co-stimulatory blockade with anti-CD154 (CD40L) has been used to prevent recurrent autoimmune diabetes in islet-allografted non-obese diabetic (NOD)/Lt mice [46].

The therapeutic potential of tolerogenic DCs in transplantation and autoimmune disease has been tested in animal models. Myeloid DCs genetically engineered to express immunosuppressive proteins (such as IL-4, IL-10, TGF-β or CTLA-4Ig) can prolong allograft survival or inhibit autoimmune diseases [47]. Likewise, treatment with tolerogenic DCs, prepared *in vitro* by treating DCs with immunosuppressive agents [23] was effective in the modulation of allograft rejection [48] and led to the amelioration of various animal models of autoimmune disorders, including diabetes [49], multiple sclerosis [50], myasthenia gravis [51] and collagen-induced arthritis [52]. Overall, tolerogenic DCs generated *in vitro* have diverse potential applications for inducing tolerance in transplantation and autoimmune disease.

Biochemical mechanisms of T-cell anergy

As mentioned in a previous section and comprehensively reviewed earlier [53•], it is probable that several forms of 'anergy' exist that have not yet been distinguished biochemically. Part of the confusion undoubtedly arises from the variety of co-stimulatory molecules that modulate the TCR response and the experimental difficulties involved in studying their effects in isolation [3]. Here, we focus on anergy induced by lack of co-stimulation rather than anergy induced by blocking cell cycle progression. The most consistent property of anergic T cells is decreased proliferation and production of IL-2 [53•]. Anergy has also been defined as an unresponsive state that can be reversed by IL-2, but it is not established that IL-2 responsiveness is an essential characteristic of an anergic T cell [53•]. Nevertheless, IL-2 responsiveness provides two useful experimental criteria, demonstrating that the anergic T cell is activated to the extent that it bears a high-affinity IL-2 receptor and confirming that it is unresponsive rather than non-viable. An important point is that anergy is only a relative measure of an immune

response. Although substantial decreases in responsiveness (>100-fold) can be achieved *in vitro*, much smaller decreases (5- to 10-fold) are also likely to have significant effects on disease progression *in vivo*.

Phase 1: induction of anergy

Which signals operate during the initial induction of anergy, and which characterize the fully-anergic state? Calcium signaling is clearly critical for the first step of anergy induction. As discussed elsewhere [54•], lack of CD28 co-stimulation correlates strongly with an unbalanced or partial form of signaling in which TCR-mediated calcium influx predominates: CD28 ligation is not itself coupled to calcium mobilization, and CD28-derived signals potentiates only those aspects of TCR signaling that do not involve calcium influx. As a consequence, treatment of T cells with calcium ionophores induces an anergic state that appears to be closely related to that induced by insufficient co-stimulation. Calcium-induced anergy is mediated primarily by nuclear factor of activated T cells (NFAT), a transcription factor regulated by the protein phosphatase calcineurin, and both NFAT activation and anergy induction are blocked by the calcineurin inhibitors cyclosporin A and FK506 [55]. During a productive activation NFAT proteins are dephosphorylated and translocate to the nucleus where they cooperate with members of the AP-1 family of transcription factors to induce the expression of T-cell activation-associated genes. Sustained small increases in intracellular calcium induce activation of NFAT proteins while failing to activate other transcription factors [56]. Induction of calcium-induced unresponsiveness correlates with expression of a new set of NFAT-dependent genes that are independent of NFAT–AP-1 cooperation and do not overlap with genes activated during productive stimulation. These anergy-associated genes encode several classes of proteins that could function as negative regulators of TCR signaling and TCR-induced transcription, thus defining a genetic program associated with reduced responsiveness [54•]. Cell hybrids produced by fusing anergic and non-anergic T cells maintain an anergic phenotype, confirming that anergic T cells express negative regulatory proteins that dominantly suppress TCR activation [57].

Phase 2: implementation of anergy

What is the nature of the block in activation in anergic T cells? There is evidence for a variety of different mechanisms, not mutually exclusive. The calcium/calcineurin-induced genetic program associated with T-cell anergy includes genes encoding phosphatases, proteases and transcriptional repressors [54•], and there is evidence that each of the corresponding mechanisms (dephosphorylation and proteolysis of signaling proteins, and direct transcriptional repression of effector cytokine genes) operate to reduce T-cell responsiveness and impose T-cell anergy.

1. Among the calcium-induced anergy-associated genes are genes encoding at least three E3 ubiquitin ligases, which mediate the selective degradation of specific signaling proteins (V Heissmeyer *et al.*, unpublished): Itch and Cbl-b, whose mutation or deletion in mice is associated with disseminated autoimmune disease, and gene related to anergy in lymphocytes (GRAIL), a transmembrane, endosome-associated RING-finger protein whose overexpression blocks IL-2 induction [58••].
2. Instead of binding activating AP-1 dimers, regions of the IL-2 promoter in anergic T cells preferentially bind to the repressor complexes CREB–CREM (cAMP response element binding protein–cAMP-responsive element modulator; [59]). Similarly, anergic cells show overexpression of Tob, which promotes enhanced binding of Smad proteins to a negative regulatory element in the IL-2 promoter [60].
3. Anergic T cells have a defect in Ras activation that correlates with deficient extracellular signal-related kinase (ERK) and Janus kinase (JNK) activity [61,62]. Activation of the small G protein Rap1 seems to be responsible, at least in part, for this block: in the absence of B-Raf, which preferentially associates with Rap1, activated Rap1 competes with Raf1 for activated Ras, thus diminishing signaling through the Ras–Raf–ERK pathway [63]. Indeed, anergy is prevented by overexpression of B-Raf in T cells [64]; however, analysis of Rap1-transgenic T cells indicates that Rap1 positively regulates TCR signaling by increasing cell adhesion [65]. Potentially, Rap1 has a dual function and its ability to signal positively or negatively might be regulated by differences in the levels and kinetics of its expression [66].
4. Anergic cells also show defects in integrin avidity, probably caused by defective phospholipase Cγ1 (PLC-γ1) activation, which results in defective integrin-mediated adhesion [19,67].
5. The src-family kinase Fyn has been implicated in maintenance of the anergic state: Fyn is hyperphosphorylated in anergic cells [63,68], and soluble dimeric MHC molecules that induce T-cell anergy displace Lck from GM1-rich membrane domains with relative enrichment in TCR-associated Fyn and poor recruitment of ZAP70 [69].
6. Finally, lack of proliferation is a hallmark of anergic T cells. Anergy-associated cell cycle arrest correlates with increased levels of p27kip1, an inhibitor of cyclin-dependent kinases, which promotes cell cycle arrest in G1 [70]. Increased levels of p27kip have been demonstrated in several *in vivo* and *in vitro* systems of T-cell anergy [70–73]. The absence of signals from CD28 and other co-stimulatory receptors, as well as signals from negative coreceptors (e.g. CTLA-4), might also promote anergy induction in T cells: for example, CD28 co-stimulation is needed for adequate downregulation

of p27kip1 through activation of phosphatidylinositol 3-kinase (PI3K)–protein kinase B (PKB) pathways [74].

Relationship between tolerogenic dendritic cells, anergic T cells and regulatory T cells

There is increasing evidence for functional interactions between tolerogenic DCs, anergic T cells and regulatory T cells. Anergic T cells are relatively long-lived and can persist *in vivo* as unresponsive cells [75]. Similar to anergic T cells, $CD4^{+}CD25^{+}$ regulatory T cells are unresponsive to TCR stimulation, although they remain responsive to IL-2 [76]. Conversely, and similar to $CD4^{+}CD25^{+}$ regulatory T cells, anergic T cells generated *in vitro* with immobilized anti-CD3 can inhibit the proliferation of responsive T cells in a manner that requires cell–cell contact [77] and, when injected, they can prolong skin and islet allograft rejection [77,78]. The active role of anergic T cells in immune suppression appears to be mediated at least partly through effects on DC function [79,80]; however, DCs can render T cells anergic, as described in a previous section. DCs from the mucosal system, which are known to be more tolerogenic than systemic DCs derived from lymph nodes and spleen, also participate in the generation of regulatory T cells by expressing high levels of IL-10 upon stimulation and priming naïve T cells to differentiate in T helper 2 (Th2) and T regulatory 1 (Tr1) directions and secrete high levels of IL-4 and IL-10 [81]. Calcium-induced anergy is also a potential means of generating regulatory T cells: it spares IL-10 expressed by Th2 and Tr1 cells, thus providing a cytokine milieu that is permissive for further autocrine generation of IL-10-producing Tr1 cells [54•]. At this stage, these observations are provocative but unconnected, as they have been obtained in different systems. Further systematic investigation is warranted, as it will undoubtedly uncover additional connections between the diverse cell types and mechanisms that maintain self-tolerance in the organism.

Conclusions

The targeted deletion of a surprising number of immune-related genes is associated with hyperproliferation of T or B cells or frank autoimmune disease. Genetically, these genes all represent negative regulators that suppress self-reactivity by enforcing negative selection of self-reactive lymphocytes, interfering with generation or function of regulatory T cells, attenuating signaling through T- or B-cell antigen receptors, or promoting apoptosis of peripheral T and B cells. Of these outcomes, T-cell anergy might represent a default genetic program, globally imposed on peripheral T cells by low-level calcium influx occurring in response to recognition of self-antigens. Controlling self-reactive cells in the periphery is of vital importance to the health and reproductive fitness of an organism, and preventing their activation would confer a significant evolutionary advantage. The variety of different mechanisms for induction and maintenance of anergy that we have described in this review could represent independent and complementary strategies, developed gradually over the course of evolution, to ensure the functional inactivation of self-reactive T cells that have escaped negative selection in the thymus. Exploiting the mechanisms of peripheral tolerance is practical and likely to be rewarding from a therapeutic point of view.

Acknowledgements

We apologize that, because of space constraints, we were unable to cite many seminal contributions from investigators in the field. Supported by an Irene Diamond Foundation grant (to FM), Spanish Ministry of Science and Technology (MCYT) grants SAF2001-3449 and Ramon y Cajal program, Spanish Ministry of Health (MSC) grant FIS 01/0896 and, Junta de Andalucia (JA) grants CTS498 and SAS165/02 (to FJC), National Institutes of Health grant AI48213 (to AR), and a Cancer Research Institute fellowship (to S-HI).

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Now in press

The work referred to in the text as (V Heissmeyer *et al.*, unpublished) is now in press [82••]:

82. Heissmeyer V, Macián F, Im S-H, Varma R, Feske S, Venuprasad K, •• Gu H, Liu Y-C, Dustin ML, Rao A: **Calcineurin imposes T cell unresponsiveness through targeted proteolysis of signaling proteins**. *Nat Immunol* 2004, in press.

This paper provides evidence for a complex and multistep program of anergy induction and implementation, which involves upregulation of the E3 ligases Itchm, Cbl-b and GRAIL during the step of anergy induction. This is followed by mono-ubiquitination, lysosomal targeting and proteolytic degradation of membrane-proximal signaling proteins phospholipase Cγ1 (PLCγ1) and protein kinase Cθ (PKCθ), and disintegration of the immunological synapse.