Cell-cycle regulation in immunity, tolerance and autoimmunity

Dimitrios Balomenos and Carlos Martínez-A.

To trigger an effective immune response, lymphocytes must proliferate. In addition to their direct involvement in cell-cycle progression, cell-cycle regulators might thus control immune functions. Recent evidence suggests that these regulators are essential for T-cell function; we argue that their study will provide clues for dissecting anergy and tolerance mechanisms, as well as for intervention in autoimmune diseases.

Following antigen exposure, mature lymphocytes require intense, prolonged and repeated proliferation to establish a rapid immune response and generate immunological memory. Proliferation also precedes induction of tolerance to soluble antigens. In addition, immune cells in bone marrow or thymus undergo repeated cycling as part of their development. It therefore appears that cell proliferation is a mandatory process for immune-system function. It has been established that deregulation of apoptosis in lymphocytes might lead to autoimmunity; this is the case for mice with defects in tumor necrosis factor (TNF) family apoptosis-related molecules such as the Fas/Fas signaling ligand system[1], as well as in Bcl-2-overexpressing[2] or Bim-deficient mice[3]. Although Fas-defective CD4$^-$CD8$^-$ T cells from MRL-*lpr* mice (a mouse substrain genetically predisposed to the development of systemic lupus erythematosus-like syndrome, which carries a mutation in the *Fas* gene) are unresponsive following *in vitro* stimulation[4], we have shown that, *in vivo*, the T cells of young MRL-*lpr* mice overproliferate, suggesting that defective apoptosis might lead to increased cell cycling[5,6]. Increased lymphocyte cycling leads to break of tolerance and autoimmunity as well as lymphoma generation[7]. Defective apoptosis, especially in conjunction with cell-cycle defects, can also lead to development of lymphomas[8,9]. Loss of tolerance and autoimmunity might thus stem from apoptosis and/or cell division defects that are also characteristic of transformation.

Studies of mice deficient in various cell-cycle regulators have shown that some of these molecules are indispensable for cell-cycle control, others might be redundant, and the requirement for growth regulators might in some cases be tissue-specific[10,11]. We and others have recently reported that regulatory T-cell functions such as anergy and tolerance appear to be dependent on cell-cycle-related molecules not required for general cell-cycle control. On the basis of these findings and the prolific lymphocyte cycling observed during the immune response, we argue for a unique role for cell-cycle regulators in controlling lymphocyte proliferation, anergy and tolerance.

Cell-cycle regulation and the immune system

Following mitogenic stimulation, quiescent cells (G0 state) progress through the four cell-cycle phases: G1, the first gap phase, S, DNA synthesis, G2, the second gap phase, and M, mitosis. Control of this process is complex, involving a large number of positive regulators such as cyclins and cyclin-dependent kinases (CDK), and negative regulators such as CDK inhibitors. These events are described below (for review, see Refs 10–12) and outlined in a simplified scheme (Fig. 1). During G1/S phase progression, cyclins D (D1–D3) act in mid-G1, followed by cyclin E and cyclin A involved at the G1/S boundary; cyclins A and B act during S and G2/M phases. CDKs require association with cyclins as well as phosphorylation for activity. CDK4 and CDK6 are associated with cyclins D, whereas cyclins A and E assemble with CDK2. The activity of cyclin–CDK complexes is repressed by CDK inhibitors, which constrain entry into S phase. On the basis of their structural characteristics and CDK targets, two classes of CDK inhibitors have been defined. p15, p16, p18 and p19 are defined as INK4 (inhibitors of CDK4) and associate solely with CDK4 and CDK6. The other group of inhibitors includes p21, p27 and p57 (the Cip/Kip family), which interfere with cycling by binding to both cyclin and CDK subunits and inhibit all CDKs involved in G1/S transition. p21 and p27 contribute to the association and activation of cyclins D with their complementary CDK, indicating that these regulators also play a positive role in controlling G1/S transition[13]. Here, we present an abbreviated outline of cell-cycle control, but this is a rather complex process that involves other important regulators including, among others, the retinoblastoma tumor suppressor protein (Rb), which acts at several control points, or the p53 protein[10–12].

Cell-cycle factors in lymphocyte proliferation and related functions

Following stimulation, lymphocytes are activated, exit G0 via a pathway dependent on the Rel/NF-κB transcription factors[14–16]. After activation, regulation of the immune-cell cycle is generally governed by the same molecules as in other cell lineages. Nonetheless, as will be discussed and as summarized in Table 1, some cell-cycle-related molecules are associated with lymphocyte proliferation in a unique manner, and control essential aspects of immune system function. This article focuses on T-cell tolerance, and emphasis will therefore be placed on cell-cycle regulation in T cells. It should nonetheless be mentioned that B cell-cycle regulation has been studied under diverse stimulation conditions[18,22,30].

Cyclins D1–D3 are expressed in cells during the G1 phase, although cyclin D1 expression is cell lineage-specific and not expressed

PII: S0167-5699(00)01748-5

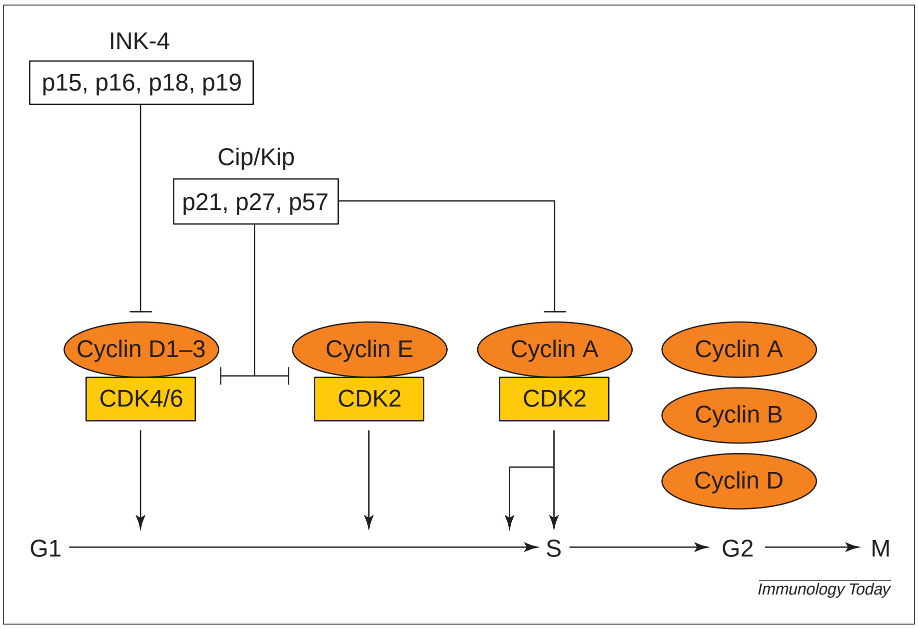

Fig. 1. *Control of cell-cycle progression from G1 to S transition. Following stimulation, cyclins D and CDK2 or CDK6 are induced and coupled, initiating the G1/S transition. Cyclin E and CDK2 drive the cell into S phase, and progression of the cycle proceeds. Cyclin A assembles with CDK2 during late G1 and early S phase. The rest of the cell-cycle is controlled by cyclins A, B and D and their associated kinases. G1 to S transition is negatively regulated by the INK4 and the Cip/Kip families of CDK inhibitors.*

in T cells[11,17]. T-cell proliferation is consequently dependent on cyclins D2 and D3, which are upregulated during early and late G1 (Ref. 17), as are CDK4 and CDK6 (Ref. 20). p27, constitutively present in quiescent cells[10], is also found in T cells prior to activation, but is downregulated during G1 after stimulation[20,25,27]. In activated T cells, IL-2 provokes loss of p27, allowing CDK activation, exit from quiescence, and progress through the G1 phase. On the other hand, IL-2 triggers upregulation of the inhibitor p21, which controls CDK activity during cycling[27]. p27 overexpression is suggested to control T-cell unresponsiveness – because Stat6-deficient T cells show a reduced proliferative response to IL-4 – but maintain high p27 expression following stimulation by this cytokine[24]. In addition, unresponsive $CD4^+$ T cells from aged mice or memory $CD4^+$ cells from BXSB lupus-prone mice retain high p27 levels, even after exposure to activation signals[28,29]. Accumulation in T cells of other inhibitors, such as p16 and p15, has also been correlated with the arrested cell growth associated with replicative senescence[21]. These data suggest that cell-cycle regulators control lymphocyte activation, and that p27 might collaborate in T-cell tolerance.

p21-deficient mice develop normally and their cells progress through the cell-cycle with no apparent abnormalities; these cells are nonetheless radiation sensitive and fail to undergo p53-dependent cell-cycle arrest following DNA damage[33,34]. Although $p21^{-/-}$ T cells respond normally to polyclonal T stimulants, they show a significant proliferative advantage over control cells following prolonged IL-2 stimulation, as well as an increased proportion of cells in the S phase of the cell-cycle[32]. Long-term cultured T cells showed no apoptosis defect after anti-CD3 treatment, but did exhibit an increased number of cells in S phase, confirming a proliferative advantage following long-term activation. In summary, it appears that although lymphocyte cell-cycle regulation is similar to that of other cell lineages, some aspects of T-cell proliferation are controlled in a unique manner.

Control of anergy, tolerance and autoimmunity by cell-cycle regulators

In addition to their involvement in cell-cycle progression, some cell-cycle regulators control mechanisms implicated in T-cell tolerance, such as anergy. As mentioned above, p27 is associated with T-cell unresponsiveness; recent work shows that, following anergy induction *via* non-productive activation of T cells in the absence of costimulation, p27 accumulates in anergic lymphocytes[26]. In these experiments, a direct relationship between p27 and T-cell unresponsiveness was clearly documented in p27-transfected T cells, in which increased levels of this cell-cycle regulator were responsible for induction and maintenance of anergy. An *in vivo* system was used in parallel to confirm that $CD4^+$ cells rendered anergic by treatment with an anti-CD40 ligand antibody maintained high p27 levels, remained tolerant following transfer to non-histocompatible mice, and did not induce graft-versus-host disease.

We have associated the p21 cell-cycle regulator with tolerance and systemic autoimmune disease[32], showing that $p21^{-/-}$ mice lose tolerance to nuclear antigens at about four months of age and that, concomitant with this event, there is an increase in memory $CD4^+$ T cells and activated B cells. We reasoned that this loss of tolerance was a result of the increased proliferative response of $p21^{-/-}$ T cells following long-term stimulation. Loss of tolerance due to p21 deficiency results in a lupus-like disease, characterized by development of anti-DNA antibodies and glomerulonephritis, mainly in female mice over seven months of age. The p21 and p27 cell-cycle regulators thus not only influence T-cell proliferation, but appear to control T-cell tolerance, a critical immunological phenomenon in transplantation and autoimmunity.

Model of tolerance regulation by cell-cycle inhibitors

Although p21 and p27 participate in tolerance induction, the mechanism by which these molecules bring about unresponsiveness to self antigens is not clear. We propose a model that incorporates these two cell-cycle inhibitors in the sequence of events thought to result in T-cell tolerance, or break of tolerance and autoimmunity (Fig. 2).

Productive T-cell stimulation *in vivo* requires an inflammatory environment that elicits costimulatory interactions from antigen-presenting cells (APC); together with T cell receptor activation, this leads to T-cell proliferation, T-cell interaction with B cells, and antibody production. Conversely, tolerance to antigen might be induced following T-cell activation under incomplete costimulatory conditions, in the absence of an inflammatory environment[35]; it has recently been proposed that presentation of an antigen by non-stimulated APC leads to tolerance[36]. Although this type of antigen presentation leads to T-cell proliferation, there is no T cell–B cell interaction, and activated cells are ultimately deleted or rendered anergic, thus establishing tolerance[35,37]. Peripheral tolerance to autoantigens might occur in an analogous manner[38]. Due to the ubiquitous presence of an autoantigen, impaired cell-cycle regulation might nonetheless lead to excessive proliferation and break of tolerance after repeated antigen presentation. Cell-cycle inhibitors are thus essential in maintaining tolerance, and appear to act at two control points: (a) p21 regulates T-cell proliferation in response to an autoantigen, and (b) p27 might be responsible for maintenance of the anergic state in autoantigen-specific T cells.

Table 1. Role of cell-cycle regulators in immune-cell function[a]

Molecule	Cell type	Result	Ref.
Cyclin D1	T	Unlike D2 and D3 cyclins, cyclin D1 is not upregulated following stimulation of primary T cells	17
Cyclin D3	B	Cyclin D3, but not D1, compensates cyclin D2 deficiency following antigen receptor stimulation	18
Cyclin A/CDK2	T, B	Rag-2 accumulation and V(D)J recombination are inhibited by the cyclin A/CDK2 complex	19
CDK2, 4, 6	T	Anti-CD3 activation of quiescent T cells results in upregulation of CDKs involved in G1/S transition	20
p15, p16	T	p15 and p16 accumulate in senescent T cells	21
p18	T, B	p18$^{-/-}$ lymphocytes show hyperproliferative characteristics, whereas p18 may contribute to terminal B cell differentiation to plasma cells	22, 23
p27	T	Stat6 controls IL-4-dependent proliferation by p27 regulation	24
p27	T	T-cell expansion by CD28 costimulation is controlled by p27 downregulation	25
p27	T	T-cell anergy depends on elevated p27 expression	26
p21, p27	T	IL-2 controls proliferation by regulating p21 and p27 levels	27
p21, p27	T	Ageing-related unresponsiveness is associated with high p21 and p27 levels	28, 29
p21	B	CD40-mediated activation upregulates p21	30
p21	Mϕ	IFN-γ-dependent cell-cycle arrest by macrophages is p21-dependent	31
p21	T	p21 deletion results in T-cell hyperproliferation, accumulation of memory T cells, loss of tolerance and systemic lupus erythematosus	32

[a]Abbreviations: B, B cell; IFN-γ, interferon γ; IL-4, interleukin 4; Mϕ, macrophage; Stat6, signal transducer and activator of transcription; T, T cell.

In the studies mentioned earlier, enhanced p21$^{-/-}$ T-cell proliferation was evident only after prolonged T-cell stimulation, a circumstance akin to repeated autoantigen presentation to T cells. In the absence of p21, prolonged exposure to antigen might thus lead to break of tolerance. Since p21 deficiency does not appear to influence overall IgG production[32], accumulation of CD4$^+$ memory T cells might represent the conversion of autoreactive cells to the memory phenotype.

p27 might control the generation of T cells anergic to self antigens, leading to tolerance (Fig. 2), as is the case of tolerance generation to non-histocompatible antigens[26]. There is nevertheless a distinction between these two models, since in tolerance to autoantigen, lymphocytes proliferate before reaching the anergic state, whereas in the graft-versus-host disease model, lymphocytes are rendered unresponsive before becoming tolerant. Despite this difference in tolerance induction, p27 might nonetheless participate in anergy maintenance by autoantigen-specific T cells. Indeed, anergic T cells generated following *in vivo* stimulation by soluble antigen are defective in production of IL-2[39,40], a cytokine involved in p27 downregulation[20,27].

It is significant that p21 deletion results in a lupus-like syndrome that is gender-linked, as is the case for the human disease; hence, the p21$^{-/-}$ mouse model might be useful in elucidating the gender bias in lupus. This model could thus aid in identifying whether female hormones are directly associated to p21, whether they affect the function of a specific immune cell type, or perhaps influence the generation and/or availability of nuclear antigens.

The major immunosuppressive drugs rapamycin and cyclosporin, which interfere with cell-cycle progression, are shown to influence p21 and p27 levels. Whereas cyclosporin results in increased p21 expression[41], rapamycin treatment appears to repress the IL-2-dependent decrease of p27 expression in T cells[27]. The effects of these immunosuppressors on cell-cycle regulators might be indirect and reflect the inhibitory effect that these drugs exert on proliferation. They are nonetheless in general agreement with the view expressed above that cell-cycle regulators control tolerance and autoreactivity, with p21 upregulation restraining T-cell proliferation and p27 accumulation resulting in T-cell anergy. Analysis of the relationship between tolerance and cell-cycle regulators might assist in understanding immunosuppression and aid in the design of new therapeutic agents for autoimmune disease.

Concluding remarks

Here, we have analysed the relationship between cell-cycle regulation in T cells and specific immune functions, with particular focus on the role of p27 and p21 in T-cell anergy, tolerance and autoimmunity. Based on these data and the special proliferative characteristics of the immune system, we consider that cell-cycle regulators have a unique effect in controlling certain immune functions. This connection between immunity and cell-cycle regulation constitutes an exciting new area of research, which might lead to a better understanding of immune system function. Finally, the effect of immunosuppressive drugs on the expression of cell-cycle inhibitors suggests that the

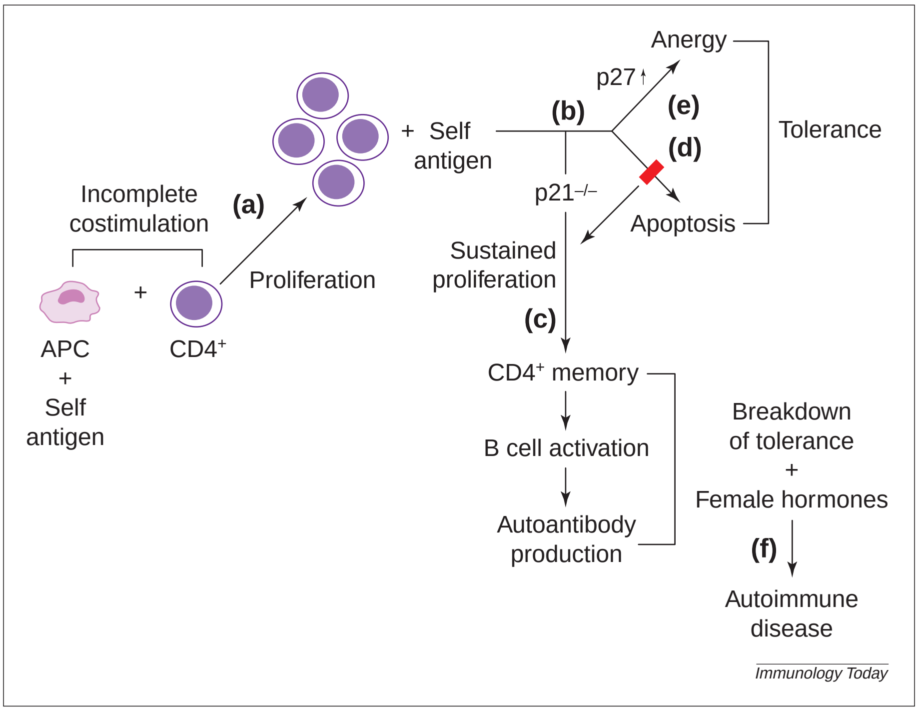

Fig. 2. *A model of cell-cycle regulation in anergy, tolerance, and development of autoimmunity. (a) T-cell proliferation following encounter with a self antigen. (b) Stimulated T cells undergo apoptosis or are rendered anergic, leading to tolerance induction. (c) In the absence of the p21 cell-cycle inhibitor, repeated encounter with antigen results in persistent proliferation, memory T-cell generation, B-cell activation, autoantibody production, and breakdown of tolerance. (d) Defective apoptosis of stimulated cells might provoke T-cell hyperproliferation and break of tolerance. (e) T-cell anergy following proliferation might be dependent on elevated p27 expression. (f) Female hormones exacerbate the tolerance defect and lead to autoimmune disease.*

study of cell-cycle regulation in this context might lead to the design of therapeutic approaches useful in the treatment of transplant rejection and autoimmunity, among other immunological syndromes.

We would like to thank María Isabel García for help with graphics and Catherine Mark for editorial assistance. The Department of Immunology and Oncology was founded and is supported by the Spanish National Research Council (CSIC) and the Pharmacia Corporation.

Dimitrios Balomenos *(dbalomenos@cnb.uam.es) and* ***Carlos Martínez-A.*** *are at the Dept. of Immunology and Oncology, Centro Nacional de Biotecnología/CSIC, UAM Campus de Cantoblanco, E-28049 Madrid, Spain.*

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Sphingolipids: second messengers, mediators and raft constituents in signaling

Eva E. Prieschl and Thomas Baumruker

Recently, evidence has accumulated to show that sphingolipids exert an important function in signaling. These lipids serve as intracellular second messengers and as extracellular mediators. Furthermore, glycosylated sphingolipids are essential components of membrane rafts, which serve as platforms for the initiation of signaling cascades. Here, Eva Prieschl and Thomas Baumruker summarize current findings in leukocytes illustrating these different facets.

By analogy to inositol (1,4,5)-trisphosphate [Ins(1,4,5)P_3] and diacylglycerol (DAG), sphingoid-based lipids (sphingolipids) were initially considered to exert their effects as second messenger molecules. This was based on the finding that DAG activates protein kinase C (PKC) (both the classical and novel isozyme variant), whereas lysosphingolipids competitively inhibit this enzyme. Cloning of endothelial differentiation genes *EDG1*, *EDG3*, *EDG5* and *EDG6*, which are specific G-protein-coupled receptors for sphingosine-1-phosphate (S1P)[1], and the secretion of S1P by stimulated platelets and mast cells, shifted attention to their 'outside in' mediator function[2] .

In the traditional model of eukaryotic membranes, lipids are considered as 'solvents' for proteins. The asymmetrical distribution of the outer versus the inner leaflet and a distinct lateral organization of the structure suggests a further role for glycosphingolipids in the function and architecture of rafts [detergent-resistant membranes, glycosphingolipid-enriched membranes (GEMs), or microdomains]. In rafts, glycosphingolipids are specifically enriched in the exoplasmic leaflet, with glycerolipids in the cytoplasmic leaflet and cholesterol in the inner spaces[3] .

Sphingolipids: second messenger molecules and 'outside in' mediators

Ceramide, a second messenger in lymphocytes

A prototype molecule for the second messenger role of sphingolipids is ceramide. The transition from regarding these molecules as membrane constituents to regarding them as signals is illustrated best in neutrophils, where ceramide accumulates during phagocytosis, particularly as ceramide-1-phosphate (C1P). Elevated levels of this phospholipid promote the fusion of liposomes, which is now attributed to the signaling character, rather than the membrane

PII: S0167-5699(00)01725-4