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The RB tumor suppressor at the intersection of proliferation and immunity: relevance to disease immune evasion and immunotherapy

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The retinoblastoma tumor suppressor (RB) was the first identified tumor suppressor based on germline predisposition to the pediatric eye tumor. Since these early studies, it has become apparent that the functional inactivation of RB is a common event in nearly all human malignancy. A great deal of research has gone into understanding how the loss of RB promotes tumor etiology and progression. Since malignant tumors are characterized by aberrant cell division, much of this research has focused upon the ability of RB to regulate the cell cycle by repression of proliferation-related genes. However, it is progressively understood that RB is an important mediator of multiple functions. One area that is gaining progressive interest is the emerging role for RB in regulating diverse features of immune function. These findings suggest that RB is more than simply a regulator of cellular proliferation; it is at the crossroads of proliferation and the immune response. Here we review the data related to the functional roles of RB on the immune system, relevance to immune evasion, and potential significance to the response to immune-therapy.

Canonical Function for RB

Multiple studies have demonstrated that RB can associate with numerous proteins across a disparate range of biological functions. However, given that RB has no catalytic activity, much of the attention has focused on how associations between RB and critical transcription factors impact transcriptional activity. These studies have taken on a particular importance given that RB is inactivated in a majority of human malignancies.[1,2] Although RB is capable of acting as a transcriptional activator[3,4] as well as a repressor, the tumor suppressive functions of RB have generally been attributed to its ability to repress transcription or otherwise modulate cell cycle progression. In this regard, the interaction between RB and the E2F family of transcription factors serves as the prototypical example of RB function. The genes involved in DNA replication, DNA repair, and G2/M progression are largely modulated by the E2F/DP heterodimer.[1] During quiescence hypophosphorylated RB masks the transcriptional activation domains of E2F/DP both directly through interaction with these proteins as well as indirectly through the recruitment of additional co-repressors. Mitogenic signals lead to the induction of CDK4/6 and CDK2 activities that promote the hyperphosphorylation of RB. This event limits RB binding to E2F proteins and co-repressors, allowing for increased transcription of genes responsible for cell cycle progression.[1] Thus the role of RB in cell cycle control is relatively well established. However, additional roles for RB exist in the regulation of immune system development and the immune response.

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Contribution to Immune Progenitor Fate Determination

The most obvious way in which RB impacts immune function is by acting as a critical regulator of transcriptional pathways at multiple checkpoints during progenitor differentiation. Haematopoietic stem cells (HSC) are multipotent progenitor cells with the capacity to differentiate into any of the haematopoietic lineages. The first decision in the process of HSC differentiation is whether the cell will become a lymphoid-lineage cell or a myeloid-lineage cell (**Fig. 1**). One of the determinants at this checkpoint in stem cell differentiation is the expression of the transcription factor PU.1.[5] PU.1 is a member of the ets family of transcription factors that is highly expressed in early myeloid lineage cells as well as specific mature myeloid populations. Thus, increased PU.1 transcriptional activity in multipotential progenitors directs these cells toward the myeloid lineage, whereas lower PU.1 activity leads these cells toward the lymphoid lineage (**Fig. 1**). PU.1 expression is still a factor in lineage determination in lymphoid cells, where cells with low levels of PU.1 will ultimately become B cells as opposed to T cells which do not seem to rely on PU.1 expression beyond very early stages. During early lineage commitment decisions, Id2, a member of the inhibitor of DNA binding family, binds PU.1 and keeps transcriptional activity in check, but ultimately this balance is maintained by mitogenic signals. In slowly proliferating cells, hypophosphorylated RB competitively binds Id2, allowing transcription of PU.1

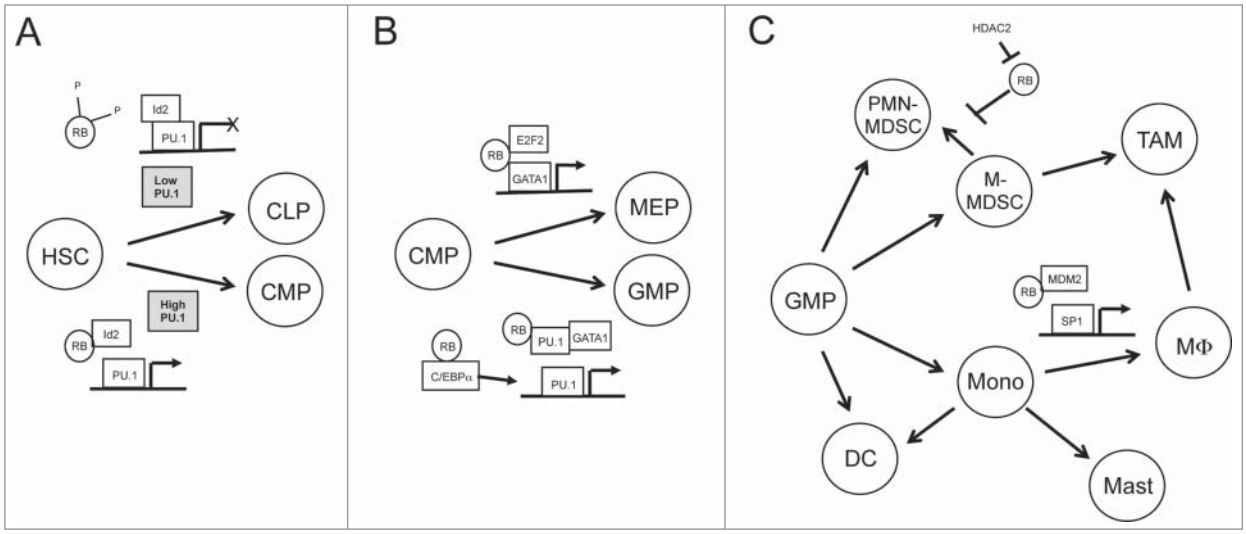

Figure 1. RB is a critical regulator of haematopoietic differentiation and immune cell development. Haematopoietic lineage fate is determined by the activity of a handful of transcription factors including PU.1, GATA-1, SP1, and the C/EBP family. RB influences the transcriptional activity of all of these factors through various interactions. Beginning at the earliest stages of haematopoietic development, (**A**) RB hypophosphorylation allows RB to competitively bind Id2, allowing for increased transcription of PU.1 target genes that favor myeloid differentiation over lymphoid differentiation. RB plays an important role in determining common myeloid progenitor (CMP) fate as well. (**B**) While RB and E2F2 promote GATA1-mediated transcription, RB also cooperates with PU.1 to repress the GATA1 program and further enhances PU.1 driven gene expression by enhancing PU.1-promoting C/EBP family members. In myeloid lineage cells, (**C**) RB contributes to myeloid cell differentiation and activation by binding MDM2, enhancing the transcriptional availability of Sp1. Meanwhile, epigenetic silencing of RB by HDAC2 skews myeloid derived suppressor cell populations toward PMN-MDSCs, which are predominantly expressed in cancer.

target genes and commitment to the myeloid lineage, whereas in more rapidly dividing cells, hyperphosphorylated RB is unable to bind Id2, resulting in commitment to the lymphoid lineage.[6]

After the initial commitment to the myeloid lineage, RB remains an important factor in determining the ultimate fate of cells (**Fig. 1**). High expression of the transcription factor GATA-1 is required for maturation of common myeloid progenitors (CMPs) into megakaryocytes or erythroid cells and this is opposed by PU.1, leading to further myeloid differentiation. In either case RB is involved in the process as GATA-1 binding to a RB-E2F2 is required for erythropoiesis,[7] but cooperation between RB and PU.1 acts to repress GATA-1 expression, blocking erythroid differentiation.[8] In addition to PU.1, further maturation of myeloid cells into macrophages or granulocytes, and the proper expression of key immunoregulatory receptors and genes in these cells, also relies on the C/EBP family transcription factors as well as Sp1. The transcriptional activity of the C/EBP family is activated through complexes with RB and, similar to PU.1, RB acts as a transcriptional activator for Sp1 by releasing the factor from its inhibitor, MDM2.[9,10] Thus, RB manages to play a critical role in the development of lymphoid, myeloid, and erythroid lineage cells at multiple points and these lineage decisions have obvious ramifications for immune function (**Fig. 1**).

Given the numerous roles RB plays in myeloid cell development, it stands to reason that alterations in the RB family may play a role in myeloid-derived cancers. Indeed, a significant number of patients with acute myeloid leukemia (AML) have either truncated RB or a total loss of RB at the protein level.[11] Similarly, RB-family deficiency has myeloproliferative effects in mouse models and inhibition of the RB-suppressor CDK4/6 impedes proliferation in vitro and increases survival in an in vivo model of Flt3-associated AML.[12,13] On the other hand, it seems reasonable that loss or RB in early haematopoietic progenitors may also skew these developing cells toward the lymphocytic lineage. This possibility seems to be supported by findings in low-hypodiploid acute lymphoblastic leukemia, where more than 40% of cases have alteration in RB.[14]

Functional Implications of RB Loss on Immune Responsiveness and Evasion

Loss of RB impacts the development of maturing leukocytes. However, this is not particularly representative of the events surrounding malignancy, where RB is functionally inactivated within the tumor but is still expressed in surrounding cell types. In this context, RB loss appears to have dual functions one of which is to influence the action of myeloid suppressor cells and influence the overall immunogenicity of the tumor.

One of the crucial functions of the immune system is detecting cellular abnormalities and clearing the aberrant cells before malignancy can develop. Thus, tumors that arise despite the normal immune response are likely to be the result of some form of immune evasion or localized immune suppression.[15] To this end, while inflammatory monocytes mature into macrophages under normal circumstances, as regulated by interactions between

RB/PU.1/C/EBPs and other factors (**Fig. 1**), myelopoiesis is skewed in cancer resulting in the increased presence of immature, activated myeloid cells known as myeloid-derived suppressor cells (MDSCs).[5,9,10,16] Expansion of these cells results in the negative regulation of immune processes including the suppression of much of the T cell response and the production of cytokines and chemokines responsible for increased rates of angiogenesis and metastasis.[17,18] As opposed to cells undergoing physiologic myelopoiesis, MDSCs in mice have been shown to not upregulate RB expression after entering the periphery from the bone marrow.[19] As a result, these cells express less RB than equivalent monocyte populations and restoration of RB expression in these cells leads to increases in traditional myeloid populations at the expense of MDSCs. This alternative pathway of myeloid development is enforced by histone deacetylase-mediated silencing of RB in the presence of tumors (**Fig. 1**).[19]

Studies with genetic deletion of RB have demonstrated the suppression of a broad range of genes associated with the immune response; including surface receptors, complement, lymphocyte factors, and cytokines.[20,21] Interestingly, this response appears to be highly evolutionarily conserved, wherein even in Drosophila models there is evidence for the suppression of genes associated with immune function in concert with RB loss.[22] The complex nature of the immune system makes it difficult to infer specifically how these alterations will affect immune recognition of resultant tumors. However, several key elements of RB loss impact Major Histocompatibility Complex (MHC), Interferon gamma response, and IL-6 signaling.

When activated, immune cells secrete cytokines that act as cell autonomous signaling mediators. These signals are generally considered to be either type 1, which stimulate cellular immune responses (e.g. IFNγ, TNFα) or type 2, which stimulate antibody responses (e.g., TGFβ, IL-6, IL-10) and proper immune function relies on the production, secretion, and recognition of these mediators to aid in recognition, uptake, presentation, and killing of pathogens and deleterious cells, such as those that would develop into tumors.[23-25] RB inhibits IL-6 gene expression and, conversely, IL-6 expression in multiple myeloma inhibits RB, leading to increased proliferation.[26,27] Similarly, in the context of RB-deficient acute myeloid leukemia, IL-6 mediates unchecked myeloid blast proliferation in an autocrine fashion.[28] Meanwhile, in mouse embryonic fibroblasts lacking RB, IFNγ is unable to stimulate MHC class II expression and this function is restored upon the addition of RB.[29] This is similar to the effect of RB loss in human tumor cell lines where it was found that RB-deficiency increases the stability of an Oct-1 containing repressosome on the HLA-DRA promoter.[30-38] The fact that this relationship could be manipulated by small molecule inhibition of RB suggests that this is an important gene regulatory network underlying tumor immunity and, presumably, the cell cycle.[39,40] Taken together, RB functions to limit type 1 cytokine signaling (IL-6) while also serving as a critical mediator of type 2 cytokine signaling (IFNγ) and MHC class II-mediated antigen presentation.

Anti-Viral Response—Is RB a Target for Immune Evasion by Oncogenic Viruses?

It has long been appreciated that RB protein is a target of various viral oncoproteins including human papilloma virus protein E7, SV40 large T antigen, and components of hepatitis B virus and hepatitis C virus.[41-45] That multiple viruses target RB suggests that RB is playing an important role in reducing the ability of these viruses to replicate efficiently. While this has previously been attributed to the loss of RB yielding enhanced capacity for DNA replication, and thus disseminating viral products more rapidly, there is mounting evidence to suggest that RB is also a key regulator of the anti-viral immune response (**Fig. 2**).

The innate immune system is designed to respond rapidly once specific molecular patterns are detected.[46] One example of this is the expression of toll-like receptors (TLRs) on antigen presenting cells. Each TLR has a defined ligand that corresponds to a particular pathogen (e.g. LPS-TLR4, ssRNA-TLR7, dsRNA-TLR3). Once a pathogenic ligand engages its cognate TLR, a series of signaling pathways are activated including those related to type I IFNs as well as NF-κB.[46] Thus, proper regulation of TLRs and their signaling pathways are crucial to recognition and clearance of pathogens and aberrant-self cells.[47] Examination of RB-deficient MEFs or cells transduced with RB-specific shRNA revealed that RB was necessary for TLR3 expression and efficient downstream signaling, including cytokine production. Meanwhile, antithetical to its typical role as a transactivator, E2F1 downregulated TLR3 expression in epithelial cells by binding to the TLR3 promoter and repressing transcription. This inhibition was initially independent of RB, but treatment with the TLR3-ligand poly(I:C) stimulated RB expression, leading to increased levels of TLR3 as a result of RB-mediated sequestration of E2F1 away from the TLR3 promoter. This positive feedback loop created by poly(I:C) and RB demonstrates the importance of RB as a regulator of immune signaling.[48]

Initial recognition of viral motifs leads to increased type I IFN production through pathways mediated by interferon regulatory transcription factors (IRFs) and NF-κB. Subsequent binding of type I IFN to its receptor results in the expression of numerous immune function genes in anti-viral gene networks. Thus, IRF/NF-κB-mediated signaling provides the first line of defense against infection by viruses. In RB-deficient cells, viral production is greater than in RB-sufficient cells, suggesting that RB serves to limit viral infectivity. This enhanced rate of viral infection is associated with decreased activation of NF-κB and reintroduction of RB results in measurable IκB degradation, allowing for the restoration of some NF-κB activity.[49] These data suggest that RB acts as a modulator of IκB stability and is required for NF-κB activity. Although loss of RB does not impact IRF3 expression, IRF5 is decreased in RB-deficient cells, suggesting that RB plays other roles in the IFN response aside from NF-κB.[21,49] Given that type I IFNs and NF-κB have numerous transcriptional targets, this implies a mechanism by which RB may greatly impact expression of numerous immune-related genes (**Fig. 2**).

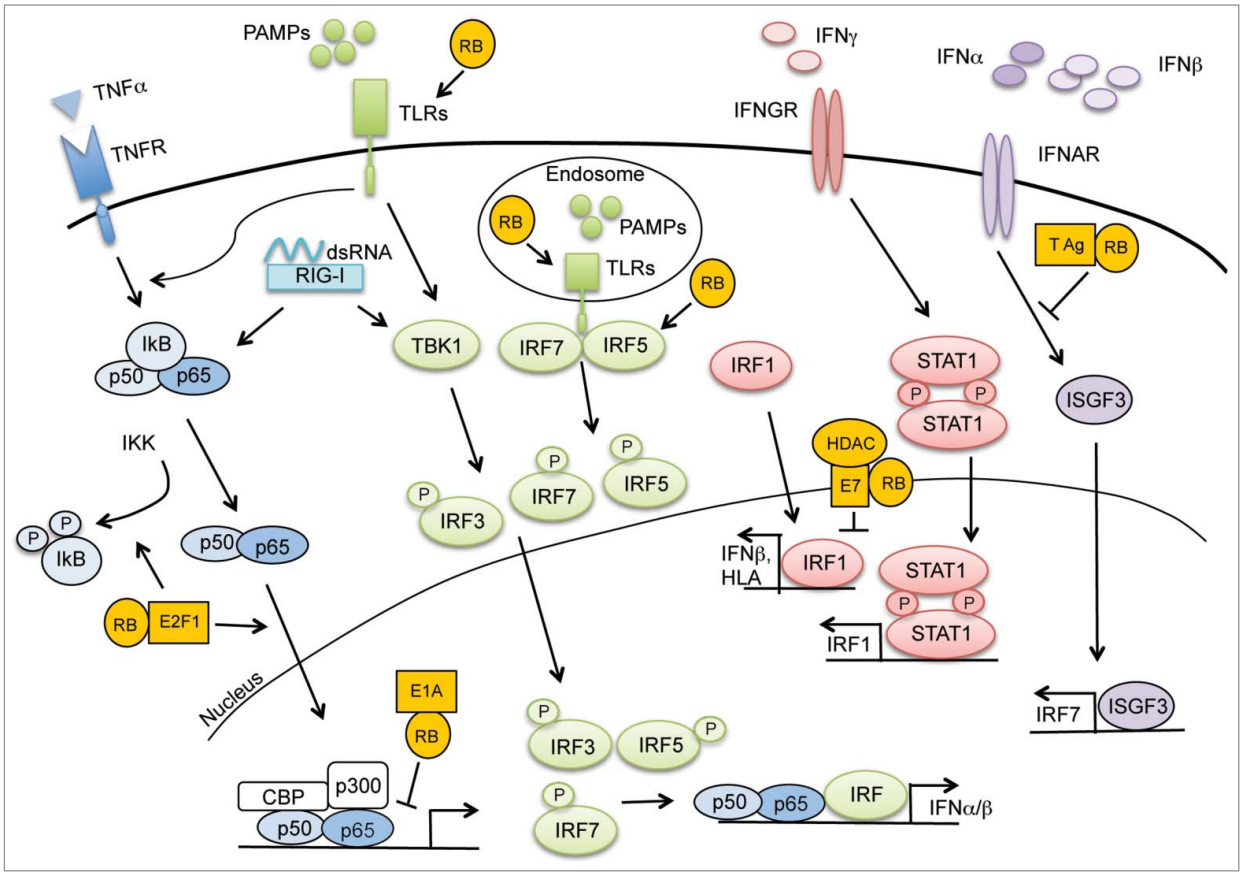

Figure 2. Anti-viral immunity requires RB. Recognition of viral motifs by mediators of the innate immune response triggers an anti-viral immune response through a variety of signaling pathways. Expression of NF-κB and IFN-mediated gene targets require RB to elicit an efficient immune response. Various viral products including E1A, E7, and Large T antigen disrupt these essential processes through their interactions with RB.

Polyoma viruses have long been recognized as having oncogenic potential at least in part because the polyoma large T antigen (T Ag) is capable of driving DNA synthesis and inhibiting cell cycle suppressors (including the RB family).[50-52] Additionally, T Ag can negatively impact the host immune response. As has previously been discussed, viral immunity relies on a robust IFN response stimulated by the recognition of viral components. Merkel cell polyoma virus T Ag promotes immune evasion via the downregulation of TLR9 expression, the receptor that mediates immune responsiveness to unmethylated CpG DNA dinucleotides.[53] Similarly, infection with murine polyoma virus elicits an inefficient immune response characterized by resistance to IFNβ and ultimately results in the development of tumors in the host. While wild-type virus is able to limit the activation of the STAT1/STAT2/IRF9 complex (ISGF3), a mutant virus lacking RB-binding capacity is incapable of mediating the same response (**Fig. 2**). This is a function of the RB-binding deficient virus being unable to bind and inhibit the STAT`1-activating tyrosine kinase JAK1.[54] Although the connection between T Ag, RB, and JAK1 remains poorly understood, this report demonstrates an immunoregulatory role for the T Ag-RB complex that would seemingly complement the proliferative impact of this association in promoting viral replication.

Although type I IFN plays an immediate role in establishing the anti-viral host response, this initial response can also result in the production of IFNγ.[55] IFNγ production can lead to the chemoattraction of numerous immune cells through the production of CXCL10 in addition to inducing increased antigen presentation on macrophages.[56] Limiting these responses is vital to the ability of viruses to evade the immune response. Given that RB plays a role in antigen presentation in response to IFNγ stimulation, it is not surprising that RB acts as a viral target in dampening the IFNγ response. Following IFNγ binding, STAT1 is phosphorylated and translocates to the nucleus where it binds interferon gamma activated sequence (GAS) domain, resulting in increased transcription of genes such as IRF1. Upon further activation of IRF1, numerous genes are transcribed including several genes associated with antigen presentation (e.g., HLA, TAP1), chemokines (e.g. CCL5), and IFNβ.[57] To counteract this process, the human papilloma virus (HPV) oncoprotein E7 transrepresses IRF-1 targets by recruiting HDAC (**Fig. 2**).[58] HPV-E7 is strongly associated with the shift from latency to cellular transformation in HPV, largely due to its affinity for host RB and the resultant increase in cellular proliferation caused by HPV-E7-mediated RB-loss. Although E7 binds HDAC independently of

RB, the RB-binding domain is necessary for the repression, suggesting that RB plays a role in this process.[58] HPV-E7 has been shown to interfere with this pathway by blocking STAT1 activation as well, although RB was neither implicated nor excluded in this association.[59]

Following the initial interferon responses to viral infection, other mediators, including tumor necrosis factor α (TNFα), provide critical support to the immune response in its efforts to eliminate viruses. There are 2 distinct outcomes following the recognition of TNFα by its receptor. One results in caspase-mediated apoptosis while the other promotes NF-κB activation resulting in increased expression of pro-inflammatory and anti-apoptotic proteins. Adenoviral infection leads to the suppression of the pro-inflammatory TNFα pathway, presumably to protect against the initiation of host immunity that would threaten viral survival and propagation even at the cost of promoting cellular death by apoptosis. To this end, it has been determined that this skewing toward TNFα-induced cellular damage and apoptosis is dependent on the formation of a complex between the adenoviral E1A protein, RB, and p300 that directly disrupts NF-κB activation at late stages in the nucleus (**Fig. 2**).[60,61] Given the functional and structural similarities with E1A, it is worth noting that E7 plays a similar role in NF-kB inhibition but does not form a ternary structure with RB and p300 and thus there is currently no evidence that RB plays a role in this association.[61]

These roles in regulating the innate immune response to viral infection have important implications in light of the paradigmatic role of RB in cell cycle regulation. Prolonged tumor progression depends on multiple factors including aberrantly increased cellular proliferation and the ability to evade immune recognition. Given that these ends coincide with the viral imperative to replicate, it is of little surprise that some viral infections ultimately result in the rise of malignancies. Indeed, it was recently shown that the pattern of decreased immune gene expression demonstrated by acute RB-loss is similar to that found in patients with advanced hepatocellular carcinoma (HCC), a disease characterized by both RB-silencing and decreased immune responsiveness at later stages. This gene signature also was associated with poor prognosis, suggesting a potential therapeutic approach for a subset of patients with advanced HCC.[21]

Impact on Immunotherapies

Given the important role for RB in limiting proliferation and the prevalence of RB disruption in malignancies, it is not surprising that RB has been an attractive target for cancer therapies. [62] As additional roles for RB in the immune response are reported, it is similarly tempting to consider how RB modulation may impact cancer immunotherapies. Recent reports offer some evidence that RB contributes in immunotherapy efficacy. Since a number of cancers result primarily from viral infections, vaccination against these viruses is perhaps the most basic form of immunotherapy. Cervical cancer, where HPV accounts for virtually all cases, is a prime example of where vaccination can reduce cancer incidence.[63] Continued development of more effective vaccines has led to the usage of DNA-based vaccines. While these have advantages over typical protein-based vaccine approaches, they tend to be less immunogenic than traditional vaccines and they also run the risk of inserting viral DNA into the host genome and, in the case of RB-associating viruses like HPV, causing unchecked proliferation. To avoid this, a DNA vaccine for HPV was designed in which the E7 protein was altered to abrogate RB binding. Not only was this vaccine stable but it was also more immunogenic than a DNA virus produced from full E7, directly implicating RB in the immunogenic response against HPV.[64]

Baccilus Calmette-Guérin (BCG) therapy was initially designed as a vaccine against tuberculosis. More recently, BCG has become a promising immunotherapy adjuvant in several malignancies including melanoma, colorectal cancer, and cervical cancer. Usage of BCG is particularly prevalent in superficial bladder cancer where instillation of BCG after tumor resection has become a first-line treatment. While the exact mechanism by which BCG triggers an immune response against bladder tumors remains unclear, RB expression predicts response to BCG.[65,66] Although these data certainly include the effects of RB alteration on proliferation, given that RB-underexpression specifically predicts BCG nonresponse when BCG is given in concert with IFNα, these reports provide evidence that RB is playing a role in the immunological response mediated by BCG instillation.[65]

These reports suggest that tumors deficient in RB could represent a particular challenge for immune-therapies. Perhaps the most well known such therapies involve immune checkpoint blockade as a result of inhibiting the function of CTLA-4 or PD-L1.[67] While expression of PD-L1 in tumor cells or tumor-associated stroma directly inhibits the cytotoxic function of CD8+ T cells by blocking co-receptor binding, CTLA-4 expression on T cells reduces the capacity of antigen presenting cells to stimulate antigen-specific responses in other T cells.[68] Although these 2 immune checkpoints have differing mechanisms, blockade of either checkpoint is intended to jumpstart an anti-tumor immune response through the restoration of T cell function. In this regard, proper function of either immune checkpoint blockade relies on TCR-MHC interaction. Similarly, a number of MHC class II-based tumor vaccines have demonstrated some efficacy in various solid tumor models based on their ability to initiate a tumor-specific CD4+ T cell response.[69-72] Given that RB is required for proper MHC expression, checkpoint blockade or other MHC class II dependent immunotherapies may be less effective in RB-deficient tumors as a result of a potentially inadequate T cell response.[21,29-39,59] Further study in the context of

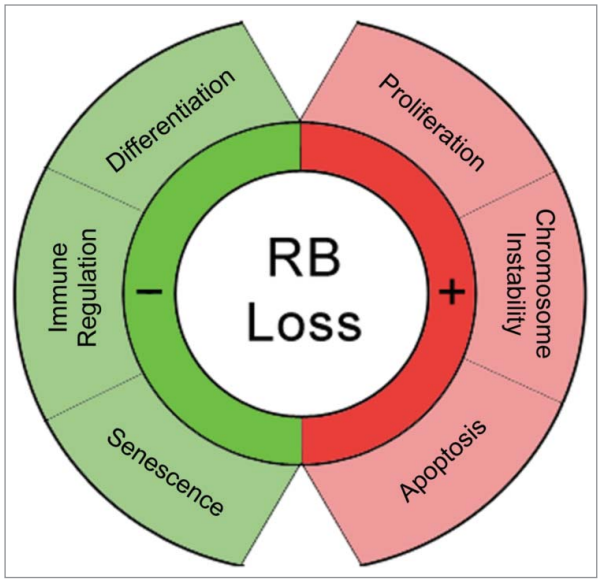

Figure 3. Loss of RB impacts multiple key functions. RB is a multi-functional protein with diverse roles in regulating cellular processes. Therapeutic interventions targeting RB should keep in mind the variety of functions that are potentially being altered.

ongoing clinical studies would be necessary to fully understand how RB loss impacts checkpoint inhibitors and other immune-based therapies.

Summary

The retinoblastoma tumor suppressor has been extensively studied, yet we still do not fully appreciate the diverse roles it plays in maintaining homeostasis by regulating proliferation, apoptosis, immunity, and development (**Fig. 3**). Although RB has been associated with the immune response for many years now, the role of RB in immunity has expanded significantly over the last few years to suggest that RB is an important mediator of innate immunity and that at least some of this functionality may be separate from the role of RB in regulating proliferation. Taken in concert, RB appears to be positioned at a key crossroad between proliferation and immunity and this can potentially be exploited in future cancer immunotherapies to improve therapeutic efficacy. Given that RB function is lost or inactivated in a majority of all human malignancies, further study is warranted to explore whether the molecular interactions that mediate the cell cycle functionality of RB and those that mediate the immune functionality of RB overlap or if it is possible to target individual aspects of RB.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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