inside blood

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HEMATOPOIESIS & STEM CELLS

Comment on Landry et al, page 5783

It takes (LMO) 2 to tango

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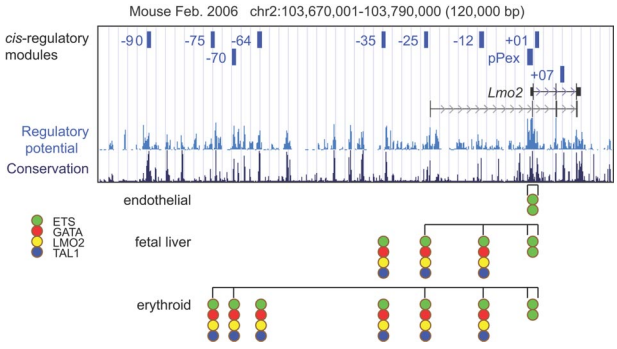
Finding gene regulatory regions and unraveling their interactions to understand tissue-specific expression are formidable challenges. Landry et al combine multiple approaches to successfully predict regulatory regions, and then show that the discovered enhancers do not work alone, but rather they work with partners to define specific patterns of expression.

Research over the past half century has shown that normal organismal development proceeds by a tightly regulated program of differential gene expression. When that regulation is lost, bad things happen. One example of considerable interest to hematologists is the ectopic expression of *Lmo2* in T cells, which leads to leukemia in some patients undergoing stem cell gene therapy regimens. Finding the DNA sequences and proteins that regulate gene expression remains a difficult challenge, but the new work from Landry and colleagues illustrates beautifully how a combination of comparative genomics and high-throughput biochemistry can identify these components efficiently.[1] The authors examine a 250-kb region surrounding the *Lmo2* gene, which is needed for hematopoiesis and vascular endothelial remodeling. They first identify17 noncoding sequences conserved from mice to marsupials, and then selected 11 with high levels of histone acetylation (assayed by hybridization of chromatin-immunoprecipitated DNA to custom microarrays) for direct functional tests in transgenic mice. From these candidates, Landry et al identify a proximal promoter, a distal promoter, and 8 distal enhancers (see figure). This is a remarkably high success rate for predictions of *cis*-regulatory modules (CRMs). Some of these had been predicted from previous studies,[2-4] but these earlier predictions also included several false positives. Searching for signatures of both evolutionary selection and histone modification–associated regulation led to more reliable predictions.



Prediction and validation of *cis*-regulatory modules for *Lmo2*. CRMs were predicted both by their conservation from mouse to the marsupial opossum (a measure of sequence constraint is shown on the last line of the locus map) and by peaks of acetylation of histone H3 in the chromatin. The CRMs with demonstrable activity in transgenic mice are shown as blue boxes on the first group of lines on the map (pPex is the extended proximal promoter), followed by the locations of introns and exons of the *Lmo2* gene and a distal promoter. The regulatory potential[3] score (light blue data track) has peaks in most of the CRMs, but some noncoding regions with strong constraint and regulatory potential are not active in the transgenic mouse assay. Below the locus maps is a summary of the occupancy of the CRMs by transcription factors and the pattern of expression that they regulate. See the complete figure in the article beginning on page 5783.

Many CRMs have been characterized by one major function, a promoter or an enhancer that increases expression in a particular tissue. Genes expressed in multiple tissues can have a specific enhancer for each tissue. Landry et al show that *Lmo2* is regulated in a different way—combinations of CRMs are needed to specify a pattern of expression. The extended proximal promoter directs strong expression in endothelial cells. However, when this promoter is combined with a set of distal enhancers, the gene is expressed in fetal liver and erythroid cells (see figure). Transcription factors of the ETS family (SFPI1 and FLI) are bound to the proximal promoter, and additional transcription factors with roles in hematopoiesis (TAL1, LMO2, and GATA2 factors) are bound to the distal CRMs. However, no single enhancer specifies erythroid expression. Just as multiple approaches improve predictions of CRMs, it takes more than one CRM to expand the expression of *Lmo2* into hematopoietic tissues.

Conflict-of-interest disclosure: The author declares no competing financial interests. ■

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Comment on Jones et al, page 5920

Stem cells in Hodgkin lymphoma?

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In this issue of *Blood*, Jones and colleagues take advantage of an important yet largely overlooked observation from 20 years ago that described the presence of small B cells in the Hodgkin lymphoma cell line, L428.

When these cells were plated out as single cells, they gave rise to pleomorphic large cells resembling what we recognize as Hodgkin Reed-Sternberg (HRS) cells. The work presented in this issue of *Blood* provides compelling evidence for the presence of clonotypic B cells in the peripheral blood of patients with classic Hodgkin lymphoma (cHL), building on this seminal observation by Newcom et al in 1988.[1]

Hodgkin lymphoma is unique among human cancers because the malignant cells typically comprise only 0.1% to 1.0% of the total cells in a biopsy.[2] Thus, most of what the clinician feels when palpating the abnormal lymph nodes of patients with Hodgkin lymphoma are non-neoplastic cells. This paucity of large neoplastic HRS cells has hampered our understanding of the nature of these cells, made it difficult to establish permanent cell lines, and precluded the development of useful animal models. The advent of laser-capture microdissection has proven instrumental in overcoming this obstacle to more detailed study of the molecular biology of this disease. Now, relatively purified HRS cells can be obtained from frozen sections of clinical samples and studied using molecular genetic and even genome-wide approaches following nucleic acid amplification.

Briefly, HRS cells in cHL are unique B cells that bear little phenotypic resemblance to any normal counterpart in the immune system. They do harbor clonal immunoglobulin (IG) heavy chain gene (*IGH*) rearrangements but fail to express surface IG and have essentially extinguished the B-cell transcription program. More recent evidence suggests that this is in part the result of epigenetic silencing.[3] Sequence analysis of the *IGH* rearrangements from HRS cells shows somatic mutations, thus linking the cell of origin to the germinal center. Typically, these cells express CD15 and CD30, but only a fraction show dim and variable CD20 expression.

Jones et al study 2 Hodgkin lymphoma cell lines, L428 and KM-H2, using flow cytometry and reconfirm the presence of a small population of $CD20^+$ B cells in these cultures.[4] Furthermore, they show that these B cells are clonal (lambda) and demonstrate phenotypic features consistent with memory B cells ($CD27^+$). Moreover, these cells also express $ALDH^{high}$, a reliable marker of stem cells/repopulating cells. Importantly, a limiting dilution strategy with the replating of these B cells supported the growth of both $CD20^+$ B cells and HRS cells, establishing that these cells can give rise to the characteristic HRS cells of Hodgkin lymphoma. Armed with this information, the authors then studied the biopsies and the matched peripheral blood specimens of Hodgkin lymphoma patients to determine whether similar cells are present. Using cell enrichment techniques, they demonstrate that a small percentage of $CD19^+$ clonal B cells can be found in the blood of patients with cHL. In addition, following sequencing of the *IGH* from both the blood and the HRS cells from biopsies, they showed that these cells are clonally identical. These data thus establish that clonotypic small B cells can be found in the blood of Hodgkin lymphoma patients and, in the small number of patients studied, this finding appeared to be independent of clinical stage. However, although the authors clearly establish that these cells are clonotypic, they were not able to demonstrate that they are clonogenic and thus have not met the burden of proof required to suggest that they constitute "cancer stem cells."

What are the next steps? First, these experiments should be validated by other investigators. Second, in vitro strategies need to be found that allow for the isolation of sufficient numbers of these clonotypic B cells, ideally from diagnostic biopsies, to enable proof-of-principle studies in immunodeficient mice demonstrating these cells are indeed clonogenic. Third, the clinical relevance of these cells will need to be studied from a number of perspectives, including their relationship to clinical outcome, their role in minimal residual disease detection, and the possibility that they might represent important targets for novel therapies. Jones et al raise the possibility of a link between their findings and the reported efficacy of anti-CD20 (rituximab) in cHL.[5] Although this may be a relevant mechanism in Hodgkin lymphoma, recent gene expression profiling studies in cHL suggest that targeting $CD20^+$ B cells in Hodgkin lymphoma may be counterproductive.[6] Further experiments will be necessary to resolve these potentially conflicting observations. Regardless of the outcome of such studies, the Jones et al article in this issue represents an important new step toward an improved understanding of the biology of the enigmatic cancer we call Hodgkin lymphoma.

Conflict-of-interest disclosure: The author declares no competing financial interests. ■

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Comment on Golden et al, page 5927

Bortezomib and EGCG: no green tea for you?

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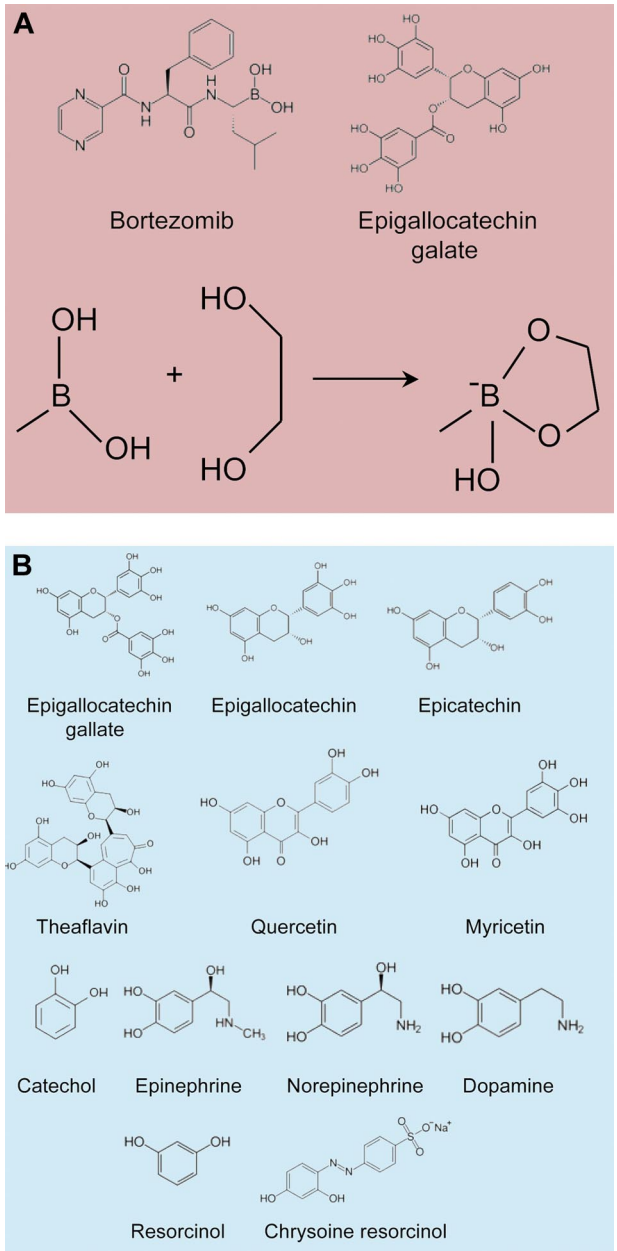
In this issue of *Blood*, Golden and colleagues report findings that indicate patients undergoing bortezomib-containing chemotherapy should avoid consuming green tea products.

Since the first description of its clinical activity against multiple myeloma and mantle cell lymphoma, the proteasome inhibitor bortezomib has become a standard of care for patients with these diseases in the relapsed/refractory setting. Modulation of proteasome function has also become an established approach to overcome chemoresistance and achieve chemosensitization in patients with relapsed/refractory and newly diagnosed myeloma, making bortezomib a crucial part of our chemotherapeutic armamentarium. Importantly, Golden et al have found that polyphenolic components of green tea, including (-)-Epigallocatechin gallate (EGCG), antagonized bortezomib in preclinical in vitro and in vivo model systems.[1] EGCG inhibited the antiproliferative effects of bortezomib on myeloma cell lines; prevented bortezomib from inhibiting the proteasome, inducing caspase-7 cleavage and activating the unfolded protein response; and protected xenografts from the proapoptotic effects of this and other peptidyl-boronate inhibitors, but not of nonboronate proteasome inhibitors. Presumably, this occurred as a result of a direct interaction leading to formation of a covalent cyclic boronate between EGCG and bortezomib (see figure), which was then no longer able to bind to the N-terminal threonine active site of the chymotrypsin-like proteasome moiety.

EGCG is only one of many polyphenols found in green tea that are classified as flavonoids, with others including epigallocatechin and epicatechin, both of which were found to inhibit bortezomib as well, although with less potency. If all compounds containing 1,2-diol groups were to have a similar activity, then black tea, which also has a number of important polyphenolic constituents including theaflavins and thearubigins, could inhibit bortezomib as well. Other compounds in this class would include myricetin and quercetin, the latter of which has already been found to bind and inhibit bortezomib.[2] Quercetin is another flavonoid that can be found at appreciable concentrations in foods, such as capers, leafy green vegetables, red onions, red grapes, red apples, and a number of berries, among other sources.[3] Interestingly, epinephrine, norepinephrine, and dopamine, all of which are derived from catechol, bear 1,2-diols, and boronates can also be bound by 1,3-diols based on resorcinol. This by no means exhaustive list clearly indicates that green tea may represent only the beginning of this story rather than its end.

Do these findings support a recommendation to patients that they avoid the use of green and black teas and flavonoid-containing foods, such as those described above, including chocolate? At least in the case of green tea, EGCG concentrations of 2.5 μM or higher were needed to see inhibition of bortezomib's activity, which were well above the maximal concentrations detected in one phase 1 trial that studied this agent's pharmacokinetics.[4] While such levels were achieved in a follow-up trial,[5] this required that patients ingest, preferably in a fasting state, large doses of Polyphenon E. This decaffeinated green tea catechin extract contained about 60% EGCG, which represents a much greater content of this polyphenol than that found in brewed green tea.[6] Alsoof note, pharmacokinetic studies of bortezomib after a standard dose of 1.3 mg/m^2 have revealed plasma concentrations up to 187.03 ng/mL,[7,8] or greater than 450 nM. In contrast, Golden et al show that bortezomib at



Flavonoids that may interact with bortezomib. (A) The structures of bortezomib and epigallocatechin galate are shown, as is the reaction by which a boronic acid and a 1,2-diol group can form a cyclic boronate. (B) The structures of epigallocatechin galate, epigallocatechin, epicatechin, theaflavin, quercetin, myricetin, catechol, epinephrine, norepinephrine, dopamine, resorcinol, and chrysoine resorcinol are shown.

20 to 40 nM is able to largely overcome the inhibitory impact of a 2.5- to 5.0-μM concentration of EGCG. These considerations argue against the possibility that even high levels of EGCG ingestion would impact upon the clinical efficacy of bortezomib.

Nonetheless, these data serve as an always timely reminder for healthcare providers of the importance of eliciting a complete history from patients and their families, including concomitant medications and over-the-counter supplements. The risks of such drug interactions are very real, whether they involve bortezomib and EGCG, bortezomib and vitamin C,[9] cyclophosphamide and curcumin,[10] or any of the other myriad possibilities. Moreover, they highlight the need for additional and careful studies along the lines of Golden et al, using physiologically relevant model systems to evaluate such possible interactions. Finally, they remind us of the words "moderation in all things" attributed to the Roman playwright Publius Terentius Afer, which should especially apply to any supplements used in the setting of chemotherapy.

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● ● ● MYELOID NEOPLASIA

Comment on Hollink et al, page 5951

Adding WT1 to childhood AML alphabet soup

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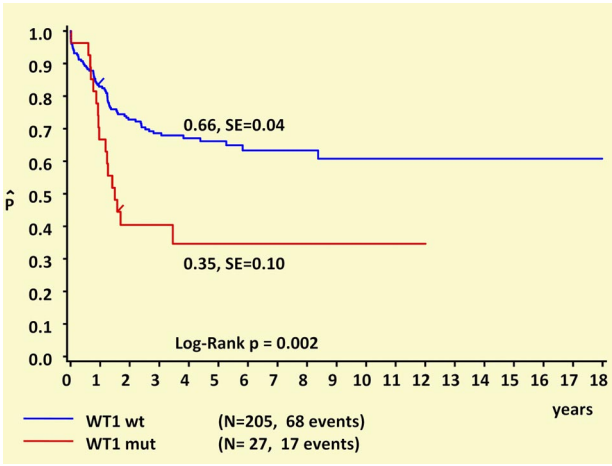
In this issue of *Blood*, Hollink and colleagues establish *WT1* mutations as a worthy addition to a growing list of molecular abnormalities that promise to improve the biologic understanding and treatment of intermediate-risk AML.

Acute myeloid leukemia (AML) is a clinically and genetically heterogeneous disease that accounts for 20% and 70% of acute leukemia in children and adults, respectively. Currently, cytogenetic analysis at diagnosis allows for stratification of AML cases into a favorable group [t(8;21), inv(16) and t(15;17)], an unfavorable group [t(6;9), abnl(3q), -7/del(7q), -5/del(5q)], and an intermediate group (normal cytogenetics or other cytogenetic abnormalities). For the favorable and unfavorable groups, risk-dependent treatment decisions, such as whether to consolidate with hematopoietic stem cell transplantation (HSCT) in first remission, have become part of standard practice. Data upon which to base such decisions have been lacking in the intermediate-risk group, however, and 60% to 70% of cases of adult and childhood AML fall into this group.

This situation has changed in recent years with the discovery of a number of molecular abnormalities that are found primarily in the intermediate-risk group and are undetectable by standard cytogenetics. Perhaps the most significant of these is the FLT3/internal tandem duplication (FLT3/ITD), since it identifies a group of patients that not only is at high risk of relapse[1] but may also benefit from novel agents that target FLT3 signaling.[2] Other important abnormalities include mutations in *NPM1*[3] and *CEBPA*,[4] each of which has been shown to identify a group of patients with a more favorable prognosis. A more recent candidate for inclusion in this list of mutations is *WT1*, which occurs in about 10% of cases of intermediate-risk adult AML and is associated with unfavorable prognosis. This article by Hollink et al is the first to fully characterize *WT1* mutations in childhood AML.[5]

A major strength of this study is the use of complementary techniques to thoroughly analyze *WT1* at the genomic and transcript levels. Thirty-five of 298 cases (12%) harbored *WT1* mutations, most of which were frameshift insertions in exon 7. In about half of the cases, biallelic *WT1* involvement could be demonstrated, due either to mutations of both alleles or to loss of heterozygosity (LOH). In cases with only one mutation, expression of both the mutant and wild-type alleles at the RNA level was confirmed, but the authors were unfortunately not able to assess expression at the protein level. Thus, the important question as to whether the wild-type protein is expressed and functional in these cases remains unanswered. This study was able to demonstrate the stability of *WT1* mutations in paired diagnostic and relapse samples, making a strong case that these mutations are a primary leukemogenic event. Moreover, *WT1* mutations were occasionally gained at relapse and may therefore represent a marker of disease progression.

Another major strength of this study is its comprehensive clinical and prognostic analysis. Like *NPM1* and FLT3/ITD mutations, *WT1* mutations are almost never seen in patients younger than 3 years at diagnosis, suggesting either a prolonged latency or an age-related resistance to the initial acquisition of these lesions. Remarkably, *WT1* and *NPM1* mutations are mutually exclusive and are both



Survival curves of childhood AML patients with and without *WT1* mutations. Kaplan-Meier estimates for 5-year pOS. See the complete figure in the article beginning on page 5951.

strongly associated with FLT3/ITD mutations. It is tempting to speculate that these cases are linked by a common underlying mutation that predisposes to DNA replication errors. What "unlinks" *WT1* and *NPM1* are their different prognostic influences. This study makes it clear that *WT1* mutations are associated with unfavorable outcomes in childhood AML, as shown in the figure. Another important difference between *WT1* and *NPM1* mutations is that *WT1* mutations retain prognostic significance in the presence of FLT3/ITD. While the favorable influence of *NPM1* mutations appears to be "trumped" by FLT3/ITD, this study suggests that the unfavorable influence of FLT3/ITD may be "trumped" by wild-type *WT1*, since the outcome for FLT3/ITD$^+$ patients who lacked *WT1* mutations was not significantly worse than for patients with wild-type FLT3.

What this study lacks is evidence supporting a causative link between *WT1* mutations and the comparatively poor response to therapy. Of particular interest would be evidence at a functional cellular level that *WT1* mutational status is associated with parameters that might predict for poor clinical outcome, such as in vitro chemoresistance or differences in apoptotic responses to DNA damage. Such findings might pave the way for *WT1* mutations to serve not only as prognostic markers but as potential molecular targets for novel antileukemic therapies.

Conflict-of-interest disclosure: The author declares no competing financial interests. ■

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● ● ● MYELOID NEOPLASIA

Comment on Ishii et al, page 5942

Itchy mast cells in MPNs

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In this issue of *Blood*, Ishii and colleagues investigate the role of mast cells in the pathogenesis of pruritus in patients with MPNs.

The BCR-ABL–negative myeloproliferative neoplasms (MPNs) of polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF) are characterized by a predisposition to thrombosis and hemorrhage, risk of blastic transformation, and premature death. In addition to these serious consequences, myeloproliferative disorder patients suffer variable constitutional symptoms, such as fatigue, bone pain, night sweats, and pruritus.[1] Data gathered from 1179 MPN patients reported that 81% suffer from pruritus, and this symptom was present in 72%, 84%, and 85% of ET, PMF, and PV patients, respectively.[1] The intensity of the pruritus can be severe, frequently exacerbated by water (ie, aquagenic pruritus), and has led some patients to discontinue bathing or to commit suicide in intractable cases. Therapy of MPN pruritus has been empiric, with antihistamines sometimes providing relief. Additionally, the use of selective serotonin reuptake inhibitors has been helpful,[2] and has suggested that platelets (the repository for serotonin in the blood) may have a role in MPN pruritus.

The pathogenesis of constitutional symptoms and particularly pruritus in MPN remains uncertain, despite recent insights into some aspects of molecular pathogenesis with identification of mutations that affect the JAK-STAT pathway including the JAK2^{V617F}, mutations in the 12th exon of JAK2, and mutations in cMPL.[3] Increased JAK2 allele burden has been associated with a greater burden of pruritus among PV patients,[4] although the exact mechanism for this association has remained uncertain, with speculation focusing on increased peripheral blood levels of cytokines. Additionally, investigators have investigated the various subsets of leukocytes that might be increased or activated in MPN patients. An association between the JAK2 mutation in basophils from PV patients, basophil activation, and degranulation with disease-associated pruritus was presented at the 2008 American Society of Hematology annual meeting.[5] Further work defining the role of basophils in the pathogenesis of MPN pruritus is ongoing.

Mast cells contain a variety of mediators of the inflammatory response (ie, histamine, tryptase, prostaglandins, and leukotrienes) that can generate pruritus. Ishii et al isolate peripheral blood mast cells as well as generating mast cells from CD34$^+$ cells of MPN patients suffering from pruritus, while not on cytoreductive therapy.[6] In their article in this issue of *Blood*, they make several salient observations. The first is that mast cells arising from MPN patients were functionally different from normal control mast cells in that they release greater levels of pruritogenic factors. Second, they observe that among patients with the most severe pruritus, (1) more mast cells are generated from CD34$^+$ cells, (2) their mast cells are less prone to apoptosis, and (3) they

have more marked increases in release of potentially pruritogenic cytokines. Finally, through use of the MPN mutation analysis, they demonstrate that mast cells from MPN patients are indeed offspring of the malignant clone, potentially explaining their functional differences from normal mast cells. These observations identify an intriguing target for therapy among MPN patients with significant pruritus, and further expand our knowledge of functional changes in leukocytes seen in MPN patients.

There is now accumulating evidence that mast cells, basophils, and even platelets may all play a role in MPN pruritus. Intriguingly, preliminary reports of JAK2 inhibitor therapy report significant decreases in pruritus,[7] and we can be hopeful that further discoveries regarding the pathogenesis of MPN pruritus may yield additional insight into the overall pathogenesis of MPNs.

Conflict-of-interest disclosure: The author declares no competing financial interests. ■

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● ● ● PHAGOCYTES & GRANULOCYTES

Comment on Lämmermann et al, page 5703

Close encounters of the 3D kind

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The small GTPase Cdc42 is a key regulator of cell polarity. In this issue of *Blood*, Lämmermann and colleagues show that DCs without Cdc42 are still able to migrate fairly efficiently on 2-dimensional surfaces but become irreversibly entangled in 3-dimensional environments, both in vitro and in vivo.

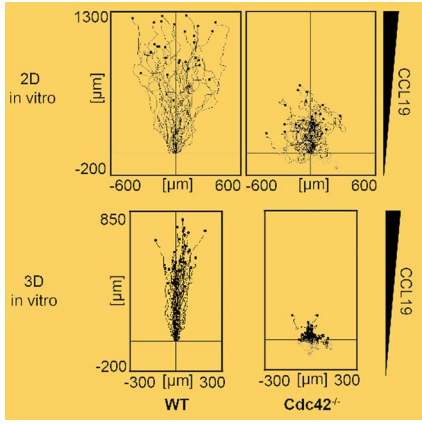
Dendritic cells (DCs) are critical for the initiation of adaptive immune responses by taking up antigen in the periphery, such as skin, to present it to lymphocytes passing through draining peripheral lymph nodes (PLNs). To perform this task efficiently, activated DCs switch their sessile sampling behavior to a highly migratory one, characterized by the acquisition of a polarized phenotype and increased expression of the chemokine receptor CCR7, which responds to its ligands CCL19 and CCL21.[1] These changes are prerequisites for efficient DC migration into afferent lymphatic vessels, which secrete CCR7 ligands and serve as a communication highway to draining PLNs.[1]

Small GTPases of the Rho and Ras families are key components of the induction and maintenance of a polarized phenotype and migration. Rac and Rho are involved in lamellipodia formation and uropod retraction, respectively. The Rho family member Cdc42 plays a role in induction and maintenance of polarity in various cell types, such as neutrophils and macrophages, in part through stabilization of the leading edge lamellipodia.[2] It remains unclear, however, how Cdc42 affected DC motility.

In this issue of *Blood*, Lämmermann et al report their findings on the role of Cdc42 during physiologic DC migration obtained in a series of elegant in vitro and in vivo assays.[3] Using primary mouse DCs derived from Cdc42-deficient bone marrow cultures, the authors investigate the migratory properties of these cells on 2-dimensional surfaces. Despite defects in maintaining polarity, Cdc42-deficient DCs still managed to migrate toward an increasing concentration of CCL19, with only slightly reduced migration velocities as compared with wild-type DCs (see top panel of figure). The residual migratory capacity was likely due to largely intact Rac-induced spreading and lamellipodia formation. Thus, on 2-dimensional surfaces, Cdc42 was not absolutely required for directed cell motility.

In a second set of experiments, Lämmermann et al examine the importance of Cdc42 during DC migration in geometrically more complex environments, that is, the 3-dimensional fibrillar networks of collagen matrices in vitro and dermis in vivo. Somewhat unexpectedly, DCs lacking Cdc42 were strongly impaired in their directed motility in 3-dimensional environments in vitro (see bottom panel of figure). Similarly, Cdc42-deficient DCs were entirely blocked in their migration from skin to draining PLNs, due to impaired entry into afferent lymphatic vessels. A more detailed morphologic analysis of Cdc42-deficient DCs uncovered that these cells became rapidly entangled within the 3-dimensional meshwork, with multiple protrusions pulling in different directions. Therefore, whereas migration efficiency in absence of Cdc42 was partially rescued due to the "lack of alternative routes" on 2-dimensional surfaces, cell motility in 3-dimensional environments absolutely required Cdc42.

Although the function of Cdc42 in other leukocytes was not addressed in this study, recent studies provide solid evidence for a



Single cell trajectories of wild-type (WT) and Cdc42-deficient DCs migrating along a CCL19 gradient in 2-dimensional (top panel) and 3-dimensional (bottom panel) settings. Despite reduced directionality, Cdc42-deficient DCs were still able to migrate with residual efficiency on 2-dimensional surfaces. In contrast, lack of Cdc42 dramatically reduced cell displacement in 3-dimensional environments due to irreversible cell entangling. See the complete figure in the article beginning on page 5703.

common integrin-independent, actin protrusion-dependent "amoeboid" migratory phenotype inside 3-dimensional environments in all hematopoietic cells.[4-6] The data presented by Lämmermann et al support the notion that directional "decisiveness" conferred by Cdc42 is a critical element of this migration mode in complex 3-dimensional settings. The observations may also explain the reduced migration of DCs deficient in the Cdc42-effector Wiskott-Aldrich syndrome protein,[7] although the more severe phenotype of Cdc42-deficient DCs reported by Lämmermann et al suggests the involvement of additional downstream effectors. Similarly, lymphocytes expressing a mutated form of the actin regulator Coronin1A, which results in excessive lamellipodia formation, show strongly impaired parenchymal motility.[8] Together with this latest report from Lämmermann et al, these findings highlight the importance of tightly controlling actin cytoskeletal dynamics for efficient "decision-making" and maneuvering through complex 3-dimensional pore systems.

In conclusion, the authors demonstrate a requirement for Cdc42 in 3-dimensional environments to avoid "cellular trapping." Their findings also highlight the importance of choosing the appropriate experimental system—in particular, 2-dimensional versus 3-dimensional settings—to dissect the physiologic role of signaling molecules orchestrating cellular motility.

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THROMBOSIS & HEMOSTASIS

Comment on Mosnier et al, page 5970

Know your APC

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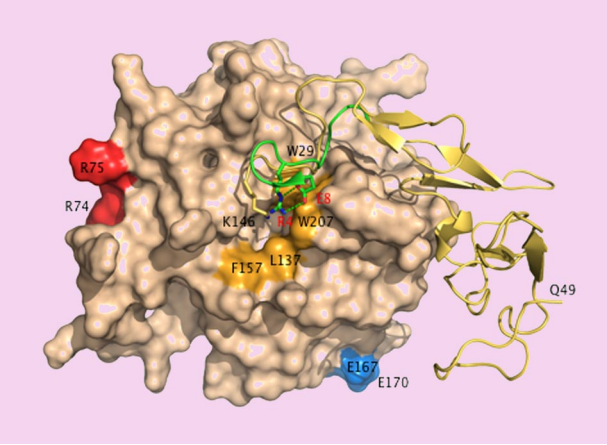
The mechanism underscoring efficacy of APC in the treatment of sepsis is still unresolved.[1] The dual nature of APC as a potent antithrombotic and cytoprotective agent complicates the task, but in this issue of *Blood*, Mosnier and colleagues offer a compelling solution and challenge the molecular underpinnings of APC function.

Ala scanning mutagenesis of the activation peptide singles out E149A, a mutant activated protein C (APC) that exhibits enhanced anticoagulant activity but greatly diminished cytoprotective effects compared with wild-type. Notwithstanding its enhanced antithrombotic activity in vivo, the variant APC is poorly effective in reducing endotoxin-induced murine mortality. Together with recent findings on a different APC variant with greatly reduced anticoagulant activity but normal cytoprotective function,[2] this important observation by Mosnier et al demonstrates that the antithrombotic activity of APC is neither necessary nor sufficient to ameliorate the outcome of sepsis.[3] It is the cytoprotective function of APC that is likely responsible for clinical efficacy, and the antithrombotic activity would only promote unwanted bleeding.

The groundbreaking discovery of the signaling properties of APC mediated by endothelial protein C receptor (EPCR)–assisted cleavage of PAR1[4] has revealed that APC is endowed with 2 distinct and physiologically important functions. APC acts as an anticoagulant by inactivating clotting factor Va with the assistance of the cofactor protein S. On the other hand, APC acts as a cytoprotective agent when it cleaves PAR1 on the surface of endothelial cells with the assistance of EPCR. Spatial separation of the underlying epitopes affords dissociation of the 2 functions by protein engineering, as previously documented in thrombin.[5] The goal is more than academic. APC variants with exclusive anticoagulant or cytoprotective activity not only provide essential reagents to dissect the functions of the enzyme in vivo, but also offer ways to improve on existing pharmacological intervention. Bleeding complications encountered in the clinical use of APC (Xigris) for the treatment of sepsis[1] could be eliminated by a variant APC that has selectively lost its anticoagulant activity.

Protein engineering of APC has already achieved important milestones. Mosnier et al have previously constructed a variant APC with greatly reduced anticoagulant activity but normal cytoprotective function.[6] The variant is as effective as wild type in reducing mortality after LPS challenge and enhances the survival of mice subjected to polymicrobial peritoneal sepsis.[3] Yang et al have recently identified a variant APC with greatly compromised cytoprotective function but normal anticoagulant activity.[7] The E149A mutant now reported by Mosnier et al further improves on the anticoagulant activity of APC in the presence of protein S at the expense of its cytoprotective function. The findings are more than a refinement of existing knowledge due to the peculiar location of E149 in the activation peptide of APC.

A patch of positively charged residues on the 30- and 70- loops (K37, K38, K39, R74 and R75) in the catalytic domain of APC provides an exosite for factor Va binding.[6] Residues E167 and E170 on the short 170-helix are important for PAR1 recognition.[7] These epitopes face the front of the enzyme and are easy targets of substrates like factor Va or PAR1 approaching the active site cleft. On the other hand, E149 is located in the back of the molecule and on the opposite side of the catalytic domain relative to the active site cleft. A fragment of the activation peptide encompassing E149 binds directly to factor Va,[8] suggesting that the epitope of this substrate extends to the back of the catalytic domain of



Surface representation of APC[15] showing the back of the catalytic domain (wheat) connected to the EGF domains and the activation peptide (yellow ribbon). The A chain of thrombin[9]-comprising residues E1c through E8 (green ribbon) is superimposed for comparison. K146 (stick) occupies a position analogous to that of R4 (stick) in thrombin. Residue E149, not resolved in the crystal structure, could engage K146 in an ion-pair interaction as E8 (stick) does with R4 in thrombin. Disruption of the ion-pair with the E149A mutation could expose a hydrophobic patch (orange) for recognition of macromolecular ligands. Residues R74, R75, E167, and E170 (chymotrypsinogen numbering) face the front of the molecule and are only partially visible in this orientation. R74 and R75 are part of the factor Va epitope.[6] E167 and E170 are involved in PAR1 recognition.[7]

APC where docking could be promoted in the presence of protein S when E149 is mutated to Ala. That would explain the enhanced anticoagulant activity of the E149A mutant. The reduced cytoprotective activity of the mutant is more difficult to rationalize in view of its unperturbed interaction with EPCR and PAR1. The activation peptide of APC folds like the A chain of thrombin, and K146, the last residue of the activation peptide visible in the crystal structure of APC, occupies the same position as R4 in thrombin.[9] The R4A mutant of thrombin has impaired activity toward chromogenic and natural substrates due to long-range perturbation of the active site.[10] Sequence alignment puts E149 in the same position as E8 in thrombin,[3] which is ion-paired to R4,[9] but the E8A mutant is catalytically compromised,[10] whereas E149A has normal activity toward chromogenic substrates and PAR1.[3]

How do we reconcile the properties of the E149A mutant with our current understanding of APC signaling? The k_{cat}/K_m value for the hydrolysis of PAR1 by APC under physiologic conditions is 0.0014 $\mu M^{-1}s^{-1}$, or more than 10 000-fold lower than that of the thrombin-PAR1 interaction.[11] How can such an insignificant rate be relevant in vivo? Colocalization of EPCR and PAR1 on the membrane of endothelial cells has been invoked to solve the conundrum,[12] but perhaps a paradigm shift is called for. APC may engage a different receptor to mediate some or most of its cytoprotective effects,[13] or APC signaling may be amplified by the release of endogenous proteases, as recently suggested for the amplification of thrombin induced PAR1 signaling in human platelets.[14] E149 and its neighbor residues may be involved in the recognition of other players besides EPCR and PAR1. The new findings by Mosnier et al remind us that we still do not know our APC.

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