inside blood

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HEMOSTASIS

Comment on Hakobyan et al, page 2060

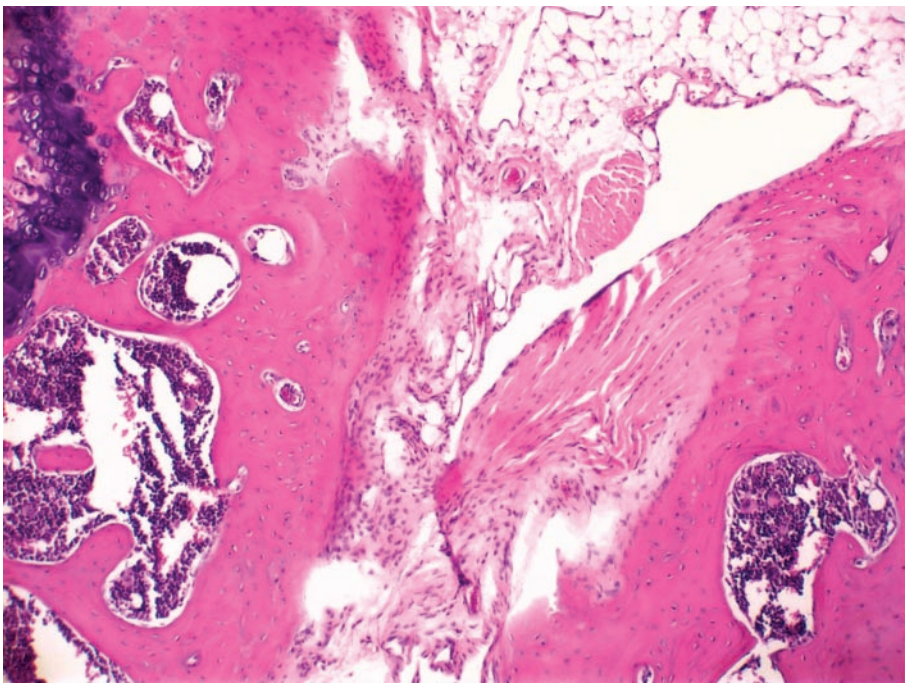
Are oncogenes responsible for hemophilic arthropathy?

Thomas Abshire EMORY UNIVERSITY

The pathogenesis of hemophilic arthropathy is not completely understood. In this issue, Hakobyan and colleagues demonstrate the association between iron, increased mdm2 expression, and synovial hypertrophy as a possible factor in this debilitating condition.

Joint disease has long been the hallmark of hemophilia, as evidenced by 2 of its most famous victims: Prince Leopold, the son of Queen Victoria, and Alexis, the young tsar-in-waiting. Current treatment with factor VIII (FVIII) or IX replacement and surgical or radioisotopic intervention has improved the lives of hemophiliacs since the days of rest, ice, compression, and elevation or the aromatic therapy that was prescribed by the charlatan monk, Rasputin. However, despite these modern advances, there have been precious few studies to help unravel the pathophysiology surrounding this debilitating condition.

Hemophilic arthropathy is characterized by synovial/endothelial cell proliferation and inflammation, though it is unclear which process is of greater importance or whether they are linked. Deep fenestrations of synovial tissue develop that remarkably resemble sea anemone and, important to this discussion, malignant tissue. Iron has long been recognized as a potential culprit in this altered pathobiology. Morris and colleagues[1] first suggested this association after describing iron deposits deep in the synovial layers. More recent studies have linked iron to both the interleukin-driven inflammatory responses involving interleukin 1 (IL-1), IL-6, and tumor necrosis factor (TNF)[2] and to *c-myc* oncogene-mediated cellular proliferation.[3]



Blood-induced synovitis as a murine model of human HS. See the complete figure in the article beginning on page 2060.

In this issue, Hakobyan and colleagues implicate the oncogene *mdm2* in the development of hemophilic arthropathy. They found that iron increases the expression of *mdm2* in human synovial cells in vitro and that increased expression of *mdm2* occurs in vivo following hemarthrosis in hemophilia A mice. The *mdm2* oncogene could affect cellular growth and death through its known direct interaction and inactivation of p53. The authors also demonstrate that genes such as *p21* and TNF-related apoptosis-inducing ligand *(TRAIL)* may be integral to synovial cell proliferation. Together, these experiments strongly implicate the role of iron in the genesis of synovial hypertrophy.

Though the important work by Hakobyan and colleagues helps to elucidate the potential role of oncogenes in hemophilic joint disease, several questions remain. Is cellular proliferation more important than inflammation in the development of joint disease? Which pathway would be best to target in interrupting the vicious cycle of joint bleeding and inflammation? Some investigators argue for the use of nonsteroidal anti-inflammatory agents to alter hemophilic arthropathy. The results reported in this issue and in other studies suggest that the development of novel agents aimed at blocking *mdm2* expression or interrupting *c-myc* expression with agents that regulate ceramide[3] could be useful. Alternatively, perhaps one

could use an iron chelator. Yet a query of equal importance ponders why all patients with severe hemophilia do not manifest joint bleeding. Approximately 10% to 15% of patients with less than 1% to 2% FVIII/IX levels effectively escape the end result of this crippling arthropathy. Clearly, it is not just iron collection in the joint cavity that causes this problem; it is iron working in concert with other mediators, perhaps protooncogenes, which appears to doom the hemophiliac to a lifetime of joint disease. Since FVIII/IX levels may not predict subsequent joint disease, increasingly, we need to separate the "bleeders" from the "nonbleeders" and perhaps intensify investigative efforts toward the latter subset to shed light on which molecular pathways or other factors of genetic heterogeneity (eg, protein C pathway) might be crucial in rendering them relatively resistant to the ravages of joint bleeding. In this world of proteonomics and gene microarrays, one can only hope that we might soon gaze into the hemophilic joint a bit more clearly. ■

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IMMUNOBIOLOGY

Comment on Flomenberg et al, page 1923

HLA-C alleles can no longer be ignored in bone marrow donor selection

Jon J. van Rood LEIDEN UNIVERSITY MEDICAL CENTRE

The central question in selecting an unrelated stem cell donor is whether we should try to match for all HLA loci and at the highest typing resolution, ie, at the allele level.

Bone marrow transplantation became a clinical reality when it was realized that HLA matching was a conditio sine qua non. In family studies, serology can be used to identify the HLA haplotypes and can thus reliably select HLA-identical sibling donors. It was soon realized that matching unrelated donor-recipient pairs only for HLA-A, -B, and -DR by serology gave suboptimal results compared with HLA-identical siblings. Furthermore, selecting unrelated donors that were negative in the cytotoxic T-lymphocyte precursor test with the patient led to better transplantation outcome and made it clear that our typing and matching procedures had been inadequate.[1] Many studies using DNA technology were started and a consensus was reached that one should attempt to find a donor who was matched at the allele level for HLA-A, -B, and -DRB1. Whether or not HLA-C, -DQ, or -DP should be included remained an open question and a matter of hot dispute.

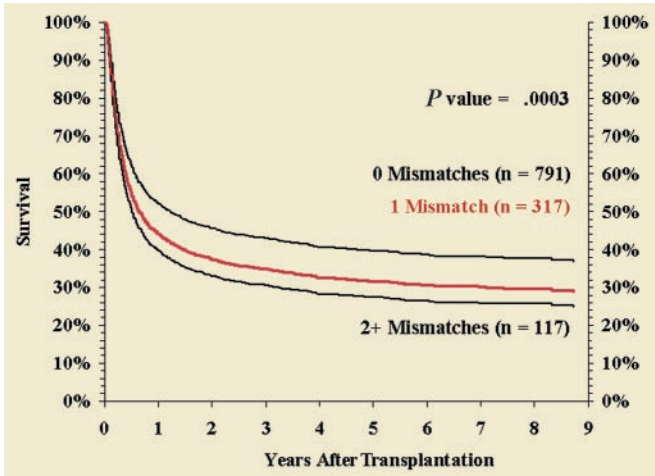
In this issue of *Blood*, Flomenberg and colleagues provide convincing evidence that one should attempt to match at the allele level not only for HLA-A, -B, and -DRB1 but also for HLA-C. The authors studied 1874 donor-recipient pairs that had all been HLA typed at the allele level for HLA-A, -B, -C, -DR, -DQ, and -DP (pairs were selected from the National Marrow Donor Program files). To assess the effect of high-resolution matching at the different loci on transplantation outcome, engraftment, graft-versus-host disease, and mortality were used as end points.

In this large study, no significant evidence was found that matching for HLA-DQ and -DP matters. Because this can only be studied adequately in HLA-A, -B, -C, and -DRB1 identical siblings, this does not imply that they are of no importance.[2]

These findings are good news for about 60% of the patients with a relatively frequent high-resolution HLA phenotype, because high-resolution HLA matching will improve transplantation outcome. Obviously, though, this is bad news for the remaining patients.

The situation is especially grim for patients carrying HLA phenotypes with a low frequency in donor registries and cord blood banks. Of the more than 9 million unrelated stem cell donors worldwide (including cord blood units), 10% have an HLA low-resolution phenotype with a frequency of fewer than 1 per million donors, so-called unique phenotypes.[3] Assuming that the HLA phenotype frequency distribution is about the same in patients and donors, it will be near impossible to find an optimally matched donor for such patients. The situation for high-resolution phenotypes will be obviously much worse. Extrapolating from available evidence, more than 20% of the patients will have such a unique high-resolution HLA-A, -B, -C, -DRB1 phenotype. Worse, we are only discussing the situation for patients with West European genetic background!

Flomenberg et al recognize this problem and emphasize that "adding more typing information to the process of donor selection should not be used to exclude more patients from the potentially curative benefits of this therapy." They also suggest that if larger numbers of transplantations could be analyzed it might be possible to identify mismatches that are more "permissible" than others.



Risk-adjusted survival among HLA-A, -B serologic, and -DRB1 allele-matched pairs by number of class I loci mismatched at the allele level. See the complete figure in the article beginning on page 1923.

Organ-sharing organizations have collected important information on permissible antigen mismatches in kidney transplantation. This was only possible because they were able to analyze files of 10 000 or more transplantations.[4] With more than 5000 transplantations per year worldwide, it ought to be possible to obtain similarly large files of stem cell transplantations with unrelated donors. Such files might provide more information not only on permissible mismatches but also on other parameters such as conditioning protocols vis-à-vis HLA mismatching, the role of natural killer (NK) cells, the use of cord blood in adult patients, and so on.[5]

This implies, however, a global effort. The study by Flomenberg et al is a good start and the forthcoming results of the 13th Histocompatibility Workshop are a good follow-up, but they regard only a fraction of the stem cell transplantations performed annually worldwide. Because stem cell transplantation poses such a high mortality risk to the patients and such a great expense to the community making it available, we have the moral obligation to attempt to realize a uniform global follow-up of stem cell transplantations. ■

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CLINICAL OBSERVATIONS

Comment on Lo-Coco et al, page 1995

Acute promyelocytic leukemia: a target for preemptive strike?

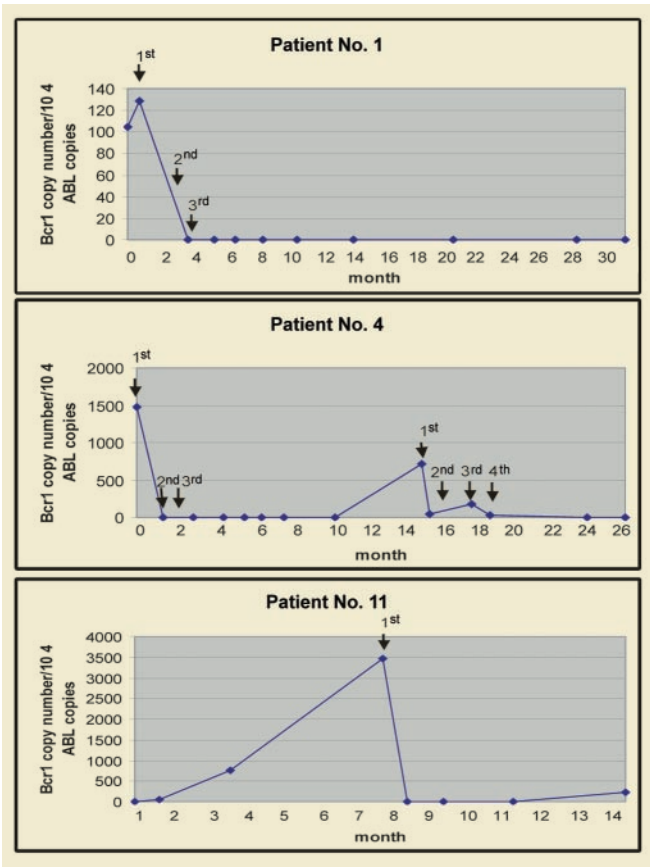
Dan Douer UNIVERSITY OF SOUTHERN CALIFORNIA

In acute promyelocytic leukemia, single-agent caliceamicin-conjugated anti-CD33 (gemtuzumab ozogamicin) is very effective in re-inducing a complete molecular remission (CRm) in almost all patients in first or subsequent molecular relapse.

In this issue, Lo-Coco and colleagues report on using caliceamicin-conjugated anti-CD33 (gemtuzumab ozogamicin [GO]) to target and treat acute promyelocytic leukemia (APL), favoring the concept[1,2] that APL cells are very sensitive to GO, perhaps more so than other acute myeloid leukemia (AML) subtypes. This was elegantly shown in patients in molecular relapse by reducing the number of copies of the APL-specific *PML/RARα* transcripts using quantitative real-time polymerase chain reaction (PCR) and reinducing them into complete molecular remission (CRm).

Consequent to development of more sensitive methods to identify minimal residual disease, new definitions of remission and relapse have emerged for AML.[3] For example, when a disease-specific gene rearrangement is known, it might be desirable to achieve the more profound CRm, such as a negative reverse transcriptase–PCR (RT-PCR). Conversely, in a molecular relapse the number of leukemia cells rises to a level detectable by RT-PCR, while the bone marrow is morphologically and cytogenetically normal and the patient is asymptomatic. A molecular relapse has special clinical significance in APL. Most APL patients in durable first CRm are cured; conversion to positive RT-PCR for *PML/RARα* almost uniformly predicts a clinical relapse within weeks or months.[4,5] Thus, CRm has become the therapeutic goal in APL. This is not the case in all AML subtypes; for example, in AML with t(8;21), RT-PCR for the *AML1/ETO* transcript could remain positive while a patient is in complete remission for years.[2]

How should clinicians respond to this molecular information in APL? Should we treat a molecular relapse preemptively (as in this paper using GO) and in what way? There are 2 potential advantages to preemptive therapy. First, re-inducing CRm prevents the potentially fatal bleeding seen in APL if treatment is delayed until a clinical relapse. Second, treatment during minimal residual disease may result in a better long-term outcome than treating overt disease with greater disease burden. The paper clearly demonstrates that to the first question: GO is very effective in re-inducing CRm in most patients in first or subsequent molecular relapse. Although 1 cycle of GO was effective in 6 of 7 patients, the minimum number of GO cycles is not clear, since most patients were not tested after the first



Results of Rt-Q-PCR studies from 3 patients during the therapy with GO. See the complete figure in the article beginning on page 1995.

cycle; however, 3 cycles seem to be sufficient. Other approaches acting by other mechanisms may also be effective, for example arsenic trioxide, which induces a very high CRm rate in overt APL relapse without the myelosuppression of GO.[6] The second potential advantage is less clear. As opposed to first CRm, only 44% of the patients in this study remained in CRm, yet in comparison to their APL historic results of overt relapse, preemptive treatment provided a survival advantage. However, this could be explained, as the authors suggest, by preventing early death of overt relapse rather than lower disease burden. Thus, GO is probably not sufficient to maintain the remission. Additional therapy such as stem transplantation or other approaches is needed. Regardless, GO is a very active drug in APL and should be further studied in combination with other treatment modalities in this disease. ■

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HEMATOPOIESIS

Comment on Zauli et al, page 2044

Stuck on the TRAIL of osteoclast differentiation

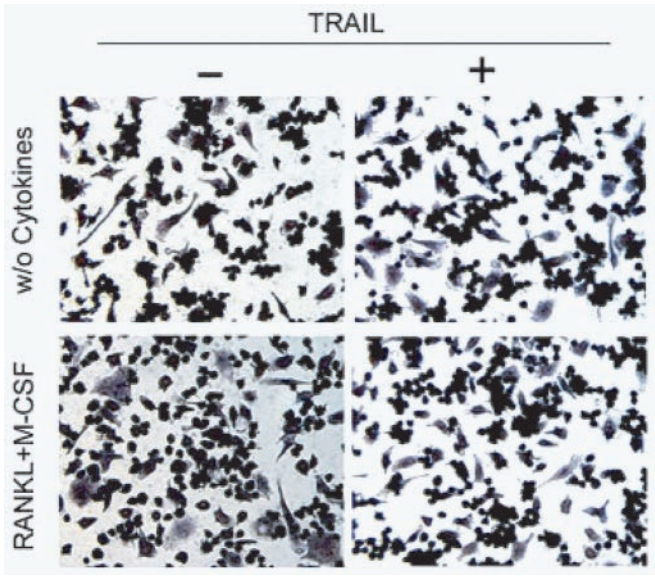
Elizabeth Olmsted Davis BAYLOR COLLEGE OF MEDICINE

Zauli et al are the first to report that the TNF family member TRAIL can inhibit osteoclastogenesis, thus suggesting a greater potential role for TRAIL in the treatment of osteolytic cancers.

Bone remodeling is an essential process for healthy maintenance of skeletal bone. However, skeletal bone is a complex system in which osteoblasts are forming bone while the osteoclasts, multinucleated cells derived from fusion of monocyte/macrophages in blood, are continually resorbing it. This process allows for the periodic replacement of bone for such purposes as removing microfissures that occur with aging and support of the kidney regulating calcium homeostasis. Therefore, it is not surprising that many pathways that lead to the production and function of 1 cell type are often controlled by the other cell type. One of the best examples of this is the tumor necrosis factor (TNF) family, which plays an integral part of osteoclast differentiation. The soluble factor RANKL (receptor activator of nuclear factor kappa B ligand) is expressed by osteoblasts and stromal cells and binds to its cognate receptor RANK on preosteoclastic cells in the local circulation, leading to osteoclast differentiation.[1] However, when the soluble receptor osteoprotegerin is present, it binds RANKL and effectively abolishes osteoclastogenesis and bone resorption.[2]

If things weren't complicated enough, in this issue of *Blood,* Zauli and colleagues demonstrate the ability of another member of the TNF family, TNF-related apoptosis-inducing ligand (TRAIL)/Apo-2 ligand (L), to inhibit osteoclastogenesis. In this report, they demonstrate the ability of recombinant human TRAIL protein to block RANKL + macrophage–colony-stimulating factor (M-CSF)–stimulated osteoclast differentiation in primary human monocytes, as well as the murine monocyte/macrophage cell line RAW264.7, thus blocking the production of the multinucleated cells and totally abolishing their bone resorptive activity.

This study is one of the first to identify a negative regulatory role for one of the TNF family members in osteoclastogenesis and bone resorption. TRAIL is best characterized for its role in inducing apoptosis in tumor cells while displaying minimal or no toxicity in normal cells.[3] Currently, no physiologic role in normal cellular processes has yet been assigned to TRAIL other than its known role in tumor protection, and mice deficient in TRAIL showed no abnormal developmental defects,[4] thus continuing the mystery. Therefore, the potential physiologic role of TRAIL as a negative regulator of osteoclastogenesis is extremely exciting, although the lack of phenotype in the gene-deficient mice suggests that there may be other factors that also can function in this capacity. The group further demonstrated that TRAIL blocked the phosphorylation of the p38/mitogen-activated protein kinase (MAPK). Since this pathway is active



Effect of TRAIL on differentiation of RAW264.7 cells into functional osteoclasts. See the complete figure in the article beginning on page 2044.

during osteoclastogenesis,[5] presumably this pathway is a regulatory target of TRAIL.

The significance of this study is, perhaps, its potential positive secondary effect of recombinant TRAIL in the treatment of osteolytic lesions such as multiple myeloma. In multiple myeloma, there is an increase in osteoclasts at the sight of lesion and a significant increase in bone resorption.[6] This uncoupling of remodeling leads to rapid bone loss and a bone degrading lesion. Thus, recombinant TRAIL may be capable of killing the tumor cells as well as attenuating the bone resorption, providing an effective treatment for this disease. ■

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CHEMOKINES

Comment on Biragyn et al, page 1961

The APC's of tumor vaccine therapy

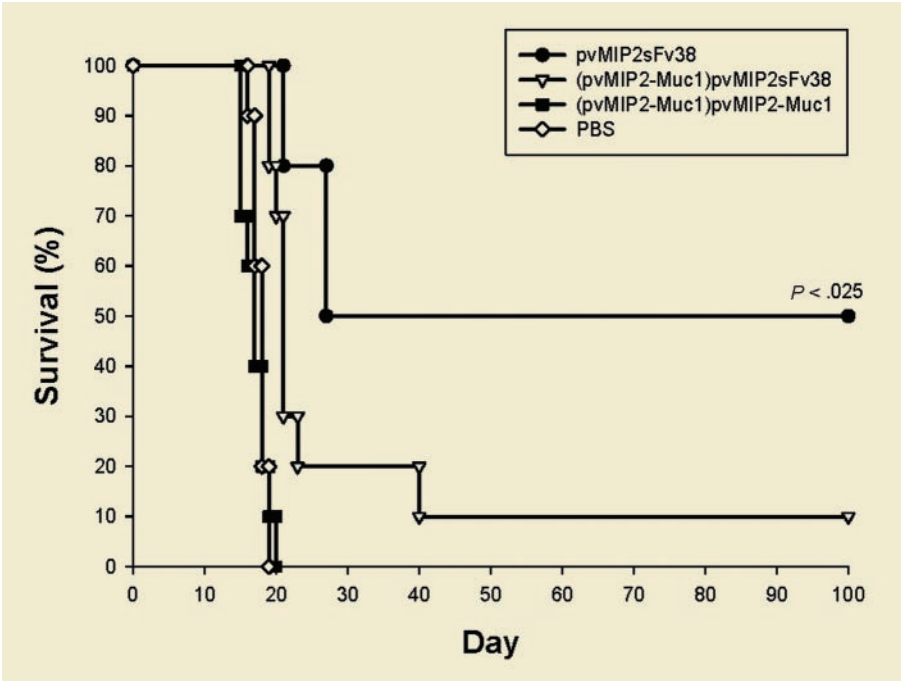
Scott S. Zamvil and Thomas Prod'homme UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

Fusion proteins containing chemokine receptor ligands have been used to selectively deliver tumor antigens to the MHC class II processing pathway in dendritic cells, promoting tumor-specific CD4 T-cell responses and tumor-specific immunity. This novel strategy of vaccination, targeting the antigen-presenting cell (APC), may have broad application to T-cell immunotherapy.

There is considerable enthusiasm in cancer therapy for developing vaccinations using protein tumor antigens or DNA-encoding tumor antigens. In fact, targeting the clonally expressed "idiotypic" antigen (Id) on malignant B cells is currently considered one of the most promising approaches.[1] However, potential lack of Id antigenicity and consequent tumor immunogenicity poses an obstacle to this approach. Tumor immunogenicity requires an effective T-cell (cellular) immune response. Previously, Biragyn et al[2,3] developed an approach to enhance the Id-specific T-cell immune response by targeting delivery of weakly immunogenic antigens to chemokine receptors. They created recombinant proteins containing proinflammatory chemoattractant ligands (chemokines and β-defensins) fused with nonimmunogenic B-cell lymphoma Id (sFv). Those chemokine-Id fusions or DNA constructs encoding the corresponding fusions, but not Id alone, induced protective and therapeutic antitumor immunity in vivo without the need for adjuvant. Results from those studies using recombinant proteins and their corresponding DNA constructs indicated that CD8 T cells participated in tumor protection, suggesting a role for major histocompatibility complex (MHC) class I–restricted antigen presentation.

In this issue, using additional fusion proteins containing chemokines whose receptors are preferentially expressed on immature dendritic cells (DCs), Biragyn and colleagues examined the role of both the class I pathway and the class II endocytic pathway in processing and presenting these proteins to T cells. Chemoattractant fusion proteins were processed and efficiently presented by immature DC populations via the MHC class II pathway to Id-specific $CD4^+$ T cells in vitro and in vivo. Compounds that disrupted the endosomal/lysosomal compartment or vesicular transport of newly synthesized class II molecules inhibited antigen processing and presentation, whereas an inhibitor of proteosomes, a structure required for delivering antigens for class I–restricted antigen presentation, did not. In vivo administration of chemokine-Id led to Id-specific tumor protection that was associated with secretion of the Th1 cytokine interferon-γ.

Evidence has supported the importance of anti-Id antibodies and CD8 T cells in antitumor responses. The use of select chemokine receptors to direct proteins into the class II processing pathway in immature dendritic cells may provide greater tumor protection. CD4 T cells are central in



Chemokine-Id fusion proteins provide efficient in vivo tumor-specific immune response. See the complete figure in the article beginning on page 1961.

importance in immune responses, not only participating as effector cells but also providing "help" for CD8 T-cell responses and humoral responses. The findings in this report raise certain questions regarding this approach. How do the internalized chemokine receptor and its ligand traffic through the endocytic pathway? Will the use of chemokine carriers elicit antihost chemokine autoimmune responses? Taking advantage of chemokine redundancy, the authors created viral chemokine-Id fusions and demonstrated that they provided equal tumor protection. However, the "non-self" carrier might be more immunogenic, limiting repeated clinical use. Clinical trials using Id vaccines have provided encouraging results.[4] Targeting the MHC class II pathway via chemokine receptors may have wide application in immunotherapy. The report in this issue, providing a method to enhance T-cell responses against a weakly immunogenic tumor, is provocative and clearly supports testing chemokine-Id fusions in clinical tumor immunity. ■

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IMMUNOBIOLOGY

Comment on Musaji et al, page 2102

Players in the sandbox of childhood ITP

Gowthami Arepally DUKE UNIVERSITY MEDICAL CENTER

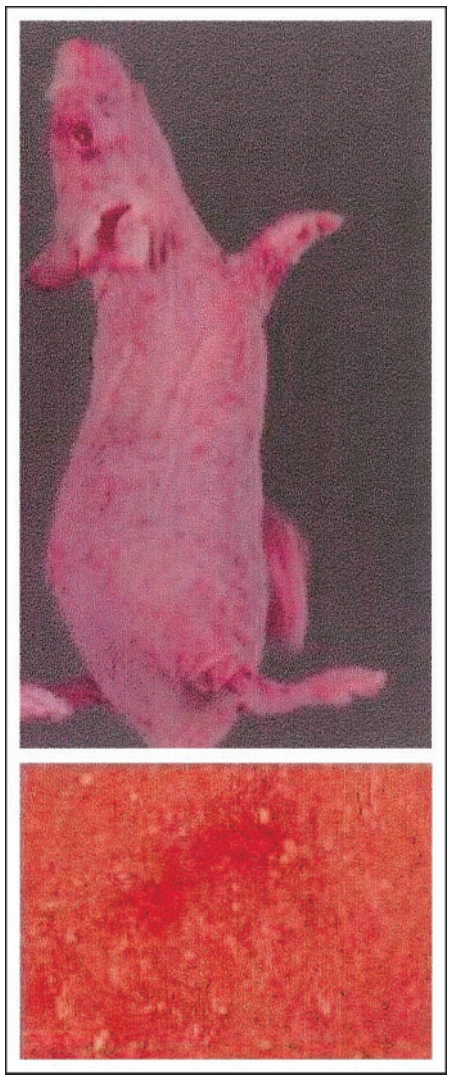
A new study examines how platelets, antiplatelet antibodies, and infection interact clinically to manifest as acute childhood ITP.

Viral infections often predate the onset of childhood or acute immune thrombocytopenic purpura (ITP), and thus they are presumed to play an essential role in the pathogenesis of this autoimmune syndrome.[1] Findings of virus-specific antibodies with cross-reactivity to platelet antigens[2,3] have advanced the notion of molecular mimicry as an instigating stimulus for childhood ITP. However, a variety of infectious agents are associated with disease onset in this syndrome,[1] and it is not known how such a diverse repertoire of infections can lead to shared immune responses to platelets. In this issue, Musaji and colleagues provide intriguing data to suggest that viral infections may not be involved in the etiology of the autoimmune response per se but rather contribute to disease pathogenesis through enhancement of host phagocyte function.

Using in vivo models, the authors demonstrate that mice infected with murine viruses alone or injected only with antiplatelet antibodies experience a moderate thrombocytopenia. However, when mice are subjected to both treatments (viral infection and antiplatelet antibodies), they develop profound thrombocytopenia within 2 to 4 days of infection, with clinical manifestations of purpura. Through additional studies, the authors demonstrate that infection exacerbates the effects of antiplatelet antibodies through interferon-γ (IFN-γ)–mediated up-regulation of phagocyte function. The authors also show that autoimmune thrombocytopenia in the setting of viral infection appears to be self-limited and does not require T-cell collaboration.

The demonstration that viruses enhance reticuloendothelial function or that phagocytic activity mediates clearance of antibody-coated platelets is hardly surprising. What is novel about this study is the experimental observation of the synergizing effects of both phenomena. The findings that mildly asymptomatic antiplatelet antibodies may produce symptomatic disease in the face of viral infection have clinical implications not only for childhood ITP but also for chronic ITP and other infection-associated thrombocytopenic disorders.

With respect to childhood ITP, the authors speculate that disease onset may occur in 2 distinctive phases: a subclinical phase in which children develop clinically inapparent platelet autoantibodies, and a thrombocytopenic phase in which enhanced phagocytic responses are triggered by viral infection. While this proposed model of disease induction may not apply to all cases of childhood ITP, especially in cases where cross-reactive viral/platelet antibodies can be demonstrated, it is a plausible explanation for the 20% to 25% of pediatric patients who appear to manifest an autoimmune-like illness.[4] For patients with adult or chronic ITP, the findings in this report also offer an explanation for profound disease exacerbations that often accompany bacterial or viral infections.[5] Lastly, this study provides some room for speculation on the mechanisms of infection-associated thrombocytopenia in otherwise healthy hosts. While bacterial and



Thrombocytopenic purpura induced by LDV infection in nude mice. See the complete figure in the article beginning on page 2102.

viral infections can elicit thrombocytopenia in a number of ways, one potential mechanism may involve unmasking of naturally occurring antiplatelet antibodies.[6] Further refinements of this experimental model will allow, perhaps, for defining the causative role of natural and pathogenic antiplatelet antibodies not only with infection but also in other inflammatory conditions associated with thrombocytopenia. ■

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NEOPLASIA

Comment on Pelletier et al, page 2163

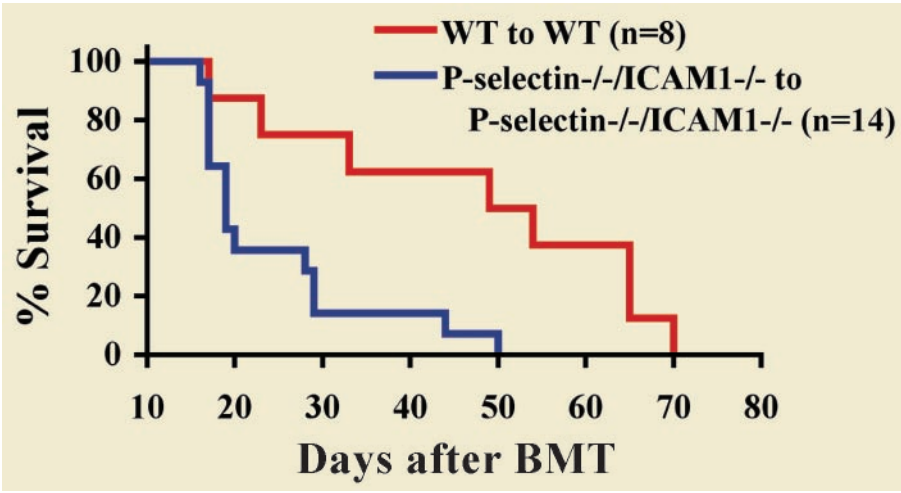
Restraining CML by adhesives

Ruibao Ren BRANDEIS UNIVERSITY

Pelletier and colleagues report that the lack of the adhesion molecules P-selectin and ICAM-1 accelerates the development of BCR/ABL-induced CML-like disease in mice. The data have important implications for understanding as well as treating CML.

Chronic myelogenous leukemia (CML) is characterized by high peripheral white blood cell (WBC) counts with granulocyte predominance and extramedullary hematopoiesis. Although the CML progenitor cells retain the ability to terminally differentiate during the chronic phase of CML, immature myeloid cells are abnormally expanded and leave bone marrow prematurely. It was postulated that, in addition to dysregulated cell proliferation and survival, altered adhesion to stromal cells and the extracellular matrix might be responsible for the abnormal circulation and proliferation of CML cells. In vitro, it was shown that CML progenitor cells from patients exhibited decreased adhesion to bone marrow stroma or fibronectin and are not subjected to normal contact inhibition of proliferation (reviewed in Verfaillie et al[1]). However, the in vivo proof of this hypothesis is lacking. Moreover, in contrast to the primary cells from CML patients, it has been found that expression of the *BCR/ABL* fusion gene, a product of the t(9;22)(q34;q11) translocation and hallmark of human CML, in hematopoietic cell lines increases instead of decreases cell adhesion to fibronectin (reviewed in Wertheim et al[2]). Interestingly, this activity of BCR/ABL is independent of the ABL tyrosine kinase activity, which is essential for BCR/ABL leukemogenesis.[3] In addition, domains of BCR/ABL, such as the ABL actin-binding domain, that are necessary for the enhanced cell adhesion are not required for the induction of CML-like myeloproliferative disorder (MPD) by BCR/ABL in mice. These conflicting results in different cells suggest that the role of cell adhesion in the pathogenesis of CML may be complicated.

In this issue of *Blood*, Pelletier and colleagues add a new dimension to the role of cell adhesion in CML. They report that the lack of the adhesion molecules P-selectin and, to a lesser extent, intercellular adhesion molecule-1 (ICAM-1) accelerates the development of BCR/ABL-induced CML-like MPD, but not BCR/ABL-induced acute lymphoblastic leukemia (ALL), in a mouse bone marrow transduction and transplantation model. The accelerated disease development in the absence of P-selectin and ICAM-1 appears to be due to altered distribution of leukemic cells; fewer of these cells are in the bone marrow and more accumulate in the lungs, rather than increased proliferation of leukemic cells. Myeloid cell infiltration and extramedullary hematopoiesis in the lungs is a characteristic of the murine model for CML and is likely the major cause of death for the diseased mice. These results suggest that cell adhesion through P-selectin and ICAM-1 plays a suppressive role in the development of CML. This conclusion has the following implications. (1) Cell adhesion through molecules such as P-selectin and ICAM-1 and negative regulation from them remain, at least partially, in CML cells. However, this study does not rule out the possibility that CML cells may have already overcome some of the negative regulations from cell adhesion. (2) Since P-selectin and ICAM-1 are not essential for homing of normal hematopoietic cells to bone marrow, loss of P-selectin and ICAM-1 must collaborate with adhesion defects caused by BCR/ABL in reducing the retention of BCR/



Lack of P-selectin and ICAM-1 accelerates the development of CML-like leukemia induced by *BCR/ABL*. See the complete figure in the article beginning on page 2163.

ABL-expressing myeloid cells in bone marrow. (3) Improving adhesion of CML cells, either enhancing normal cell adhesion or targeting BCR/ABL signaling pathways that disrupt cell adhesion, or both, may be a valuable strategy for CML therapy. Further studies are needed to demonstrate the relevance of the tumor suppression effects of P-selectin and ICAM-1 to human CML. In particular, development of pulmonary hemorrhage is a major difference between human CML, in which pulmonary manifestations are rare, and the murine model for CML. Current data could not rule out the possibility that increased accumulation of leukemic cells in the lungs in the absence of P-selectin and ICAM-1 may be due to certain experimental condition(s) of the model system. Nevertheless, this report raised an interesting possibility that normal cell adhesion may restrain CML. ■

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RED CELLS

Comment on Huang et al, page 2176, Yamaji et al, page 2178, and Matthes et al, page 2181

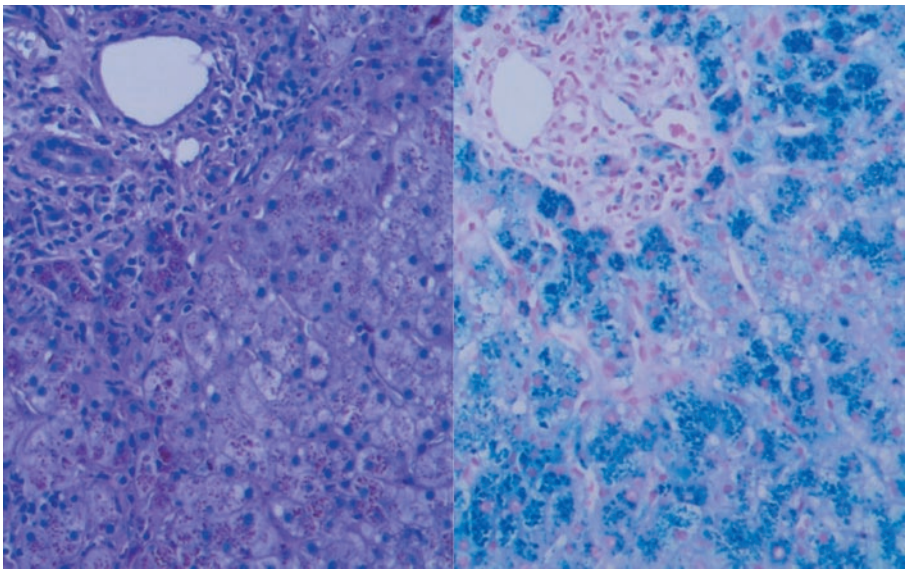
Hemojuvelin and hepcidin: the keys to JH?

Eric Nisbet-Brown THE UNIVERSITY OF TORONTO

Three papers published in this issue of *Blood* provide important insights into the pathogenesis of juvenile hemochromatosis. Two papers identify novel mutations in the genes encoding hemojuvelin and hepcidin, respectively, while the third shows a direct of hepcidin upon iron uptake by gut epithelial cells.

Juvenile hemochromatosis (JH) is a genetically heterogeneous, autosomal-recessive disorder of iron transport and storage. This rare condition is associated with accumulation of parenchymal iron in the second and third decades of life as a consequence of increased gastrointestinal absorption and decreased macrophage storage, and leads to iron-associated cardiac disease, hypogonadotropic hypogonadism, diabetes, and arthropathy.[1]

Many patients with JH have disease linked to chromosome 1q. The chromosome 1q–associated JH gene has recently been cloned and is called *HFE2* or *HJV*; its protein product is called hemojuvelin. Mutations in this gene have been shown to be associated with the JH phenotype.[2] In addition, loss-of-function mutations in the *HAMP* gene, which encodes the liver protein hepcidin, also have been shown to be associated with the JH phenotype.[3] These papers extend these findings and suggest possible linkages between hemojuvelin and hepcidin that may account for the development of JH.



Liver biopsy. See the complete figure in the article beginning on page 2176.

Huang and colleagues describe a novel mutation in *HJV* that results in a premature termination codon at position 321 of the hemojuvelin protein, leading to truncation of the protein prior to its putative transmembrane domain. They also report a missense mutation in *cis* in the hemojuvelin signal peptide, although this mutation may be functionally insignificant. The proband in their study also expressed a previously described missense mutation (I281T) in *HJV*.[2] The coexpression of the *HJV* mutations presumably caused loss of function of the hemojuvelin protein and led to severe parenchymal iron overload by the age of 19 years in this patient.

In the second paper, Matthes and colleagues describe 2 Portuguese siblings with JH who are homozygous for a previously unreported mutation in the 5′-untranslated region (UTR) of the *HAMP* gene, which results in a new initiation codon and a frame shift leading to the production of an abnormal protein. No normal hepcidin could be detected in the patients' urine. Both siblings also were heterozygous for the H63D mutation in the *HFE1* gene, although it seems unlikely that this increased the severity of the JH phenotype. The very different clinical presentations of the 29-year-old proband and his 24-year-old sister, despite possessing the same *HFE1* and *HAMP* mutations, suggest the existence of additional modifying features that affect the expression of the iron overload phenotype.

In the third paper, Yamaji and colleagues describe a direct inhibitory effect of hepcidin on iron uptake in vitro by human intestinal epithelium Caco-2 cells. Hepcidin decreases apical iron uptake and decreases expression of iron-responsive element-regulated form of divalent metal transporter 1 (DMT1[+IRE]) mRNA and protein levels but has no effect on iron efflux and iron-regulated gene 1 (IREG1) expression. These studies have several limitations, not least that the naturally occurring

concentrations of hepcidin in biologic fluids are largely unknown; these investigators used an arbitrary concentration of 10 μM and did not show dose-response kinetics. Similarly, the hepcidin preparation used in their studies included a number of different hepcidin species, and it is unclear which are active form(s).

Where do these studies lead? Studies in genetically modified mice and clinical observations in humans suggest that hepcidin plays a key role in the physiologic regulation of iron storage. Overexpression of hepcidin (eg, in states of chronic inflammation) leads to an iron-unresponsive anemia that resembles the anemia of chronic disease. Conversely, genetic deficiencies of hepcidin lead to JH.[4]

Hemojuvelin is the other key player in this scenario. The majority of characterized cases of JH have involved mutations in *HJV*/hemojuvelin, yet the function of this gene remains unknown. Hepcidin levels are decreased in patients with *HJV* mutations;[2] loss-of-function mutations in hepcidin lead to JH; and *HJV*- and *HAMP*-associated JH are clinically indistinguishable. It is therefore tempting to speculate that hemojuvelin and hepcidin are linked in a common pathway that regulates iron storage. Hemojuvelin would regulate the levels of hepcidin, the latter being the key effector protein in iron storage. The studies of Yamaji et al suggest a mechanism by which hepcidin may regulate iron uptake by intestinal epithelial cells. However, considerable further work will be necessary to confirm such a pathway and to define the physiologic mechanisms that control iron uptake and storage. The relationship between hemojuvelin/hepcidin and the *HFE1* pathway responsible for late-onset Northern European hereditary hemochromatosis also will need to be established. Finally, it will be necessary to define genetic and nongenetic modifiers of iron accumulation to account for the observed wide variation in JH clinical phenotype.[5] ■

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NEOPLASIA

Comment on Abi-Habib et al, page 2143

Site-specific conversion of a pro-fusion protein toxin

John R. Murphy BOSTON UNIVERSITY SCHOOL OF MEDICINE

Substituting the native furin endoprotease activation site with a urokinase plasminogen activator recognition sequence, Abi-Habib and colleagues have constructed a diphtheria toxin–related granulocyte-macrophage colony-stimulating factor fusion protein toxin with increased utility and specificity toward acute myelogeneous leukemic cells.

Protein engineering of bacterial protein toxins has been focused largely on substitution of the native receptor-binding domain of the toxin with surrogate ligands in order to selectively redirect toxicity toward those cells that display the appropriate surface target receptor. While there has been extensive work in this field, to date the only fusion protein toxin that has been approved for human clinical use is diptheria fusion protein targeting interleukin-2 receptor (DAB_{389}IL-2 [ONTAK]).[1-3] Abi-Habib and colleagues, in their current article, have taken a significant step forward in the field of therapeutic fusion toxins. In this instance, replacement of the native diphtheria toxin receptor-binding domain with granulocyte-macrophage colony-stimulating factor (GM-CSF) targets the fusion protein toxin toward the GM-CSF receptor on acute myeloid leukemia (AML) blasts. While this genetic construct is highly toxic toward AML cells in vitro, the normal distribution of the GM-CSF receptor in vivo is sufficiently broad that a second layer of specificity was engineered into the fusion protein toxin.

The diphtheria toxin–related fusion protein toxins follow a highly defined route of entry into the cell.[4] The intoxication process begins with receptor binding; the fusion protein toxin is then internalized into the cell by receptor-mediated endocytosis. In the lumen of the early endosome, the endoproteinase furin introduces a "nick" in the α-carbon backbone in a protease-sensitive loop that connects the catalytic (C) and transmembrane (T) domains. This nicking event is essential for the subsequent membrane translocation and release of the C-domain into the cytosol of target cells. By replacement of the furin recognition site (RVRRSV) with the urokinase plasminogen activator (uPA) cleavage site (GSGRSA), Abi-Habib and colleagues have constructed a "pro-fusion protein toxin." Their study clearly shows that the targeted action of the modified construct, DTU2GMCSF, strongly correlates with the combined presence of the uPA receptor, uPA, and the GM-CSF receptor.

The development of new fusion protein toxins as potential therapeutic agents is still in its infancy. The challenge remains to construct biologics that retain a high level of specificity toward cell surface determinants that are largely restricted to malignant cells in order to minimize damage to normal cells. Since uPA is often up-regulated in particular malignancies, the introduction of the uPA cleavage site into DTU2GMCSF confers a second level of specificity and shifts the balance of action toward the malignant cell population. We await with interest the initiation of and findings from clinical studies with this pro-fusion protein toxin as we anticipate that the coupling of cell-specific drug delivery with site-specific conversion from protoxin to toxin will greatly expand the utility and therapeutic margin of these agents. The DTU2GMCSF described by these authors offers a promising, novel, therapeutic approach for patients with AML resistant to standard treatment. ■

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TRANSPLANTATION

Comment on Miura et al, page 2187

Immunoregulation in human bone marrow transplantation?

Matthias Edinger UNIVERSITY HOSPITAL REGENSBURG

Quantification of Foxp3 messenger RNA was used to evaluate the reconstitution of the regulatory T-cell compartment after bone marrow transplantation.

$CD4^+$ T cells that constitutively express the alpha chain of the interleukin 2 (IL-2) receptor (CD25) are enriched in thymus-derived suppressor cells that preferentially express Foxp3, a forkhead/winged-helix transcriptional regulator.[1] In the study presented by Miura and colleagues, Foxp3 mRNA was quantified in mononuclear cells (MNCs) to monitor regulatory T-cell reconstitution after autologous or allogeneic bone marrow transplantation (BMT). The authors detected lower Foxp3 expression levels in patients with graft-versus-host disease (GVHD) compared with patients without GVHD after allogeneic BMT as well as in breast cancer patients developing an autoimmune syndrome (autologous GVHD) that was purposely induced by the administration of cyclosporine A after high-dose chemotherapy. Diminished Foxp3 expression correlated with a low number of T-cell receptor excision circles (TRECs) in MNCs from GVHD patients, suggesting that regulatory T cells regenerate from thymic precursors and that thymic dysfunction in autologous and allogeneic GVHD results in an impaired output of suppressor T cells, thereby perpetuating disease. Interestingly, response to GVHD therapy was accompanied by a recovery of the $Foxp3^+$ T-cell pool in a limited number of serially examined patients.

This is the first report suggesting that suppressor cell–mediated regulation may play an important role in human GVHD pathophysiology. Although the results should be interpreted with caution, since a mere positive or negative correlation does not prove (or disprove) the biologic relevance of an observed phenomenon, they are well supported by several animal studies demonstrating a central role for regulatory T cells in the development and maintenance of peripheral tolerance to self-antigens and alloantigens.[2] Sakaguchi and colleagues[3] initially revealed the significance of $CD4^+CD25^+$ regulatory T cells for the protection from autoimmune diseases in mice that underwent thymectomy early in life. Similarly, adoptively transferred rat $CD25^+$ regulatory T cells protect from autologous GVHD caused by cyclosporine treatment of syngeneic BM recipients.[4] In murine models of allogeneic BMT, Johnson et al[5] demonstrated that $CD4^+CD25^+$ regulatory T cells develop from bone marrow–derived T-cell precursors and depend on thymic maturation. Once generated, they modulate GVHD induced by the delayed infusion of donor lymphocytes.[6] Furthermore, several groups reported that the cotransplantation of large numbers of donor-type $CD4^+CD25^+$ regulatory T cells at the time of BMT protects recipients from lethal GVHD induced by nonregulatory T cells.[6] Thus, there is increasing evidence for T-cell–mediated immunoregulation after allogeneic BMT, and the findings of Miura et al should encourage clinicians to further explore those mechanisms for the development of improved hematopoietic stem cell transplantation strategies. ■

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