inhibitor, combination studies with other agents, and, if prolonged survival is seen, secondary transplant experiments. These studies will be critical in determining if the identification of CDK6 as a potential target of NUP98-FP–induced leukemia will translate into a new therapeutic approach. Even without this preclinical data, however, the authors have provided a fascinating approach to identifying such targets that should be amenable to use with other, uncommon or rare molecularly defined subtypes of AML.

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

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IMMUNOBIOLOGY AND IMMUNOTHERAPY

Comment on Roex et al, page 455

Antitumor immunity and T-cell avidity

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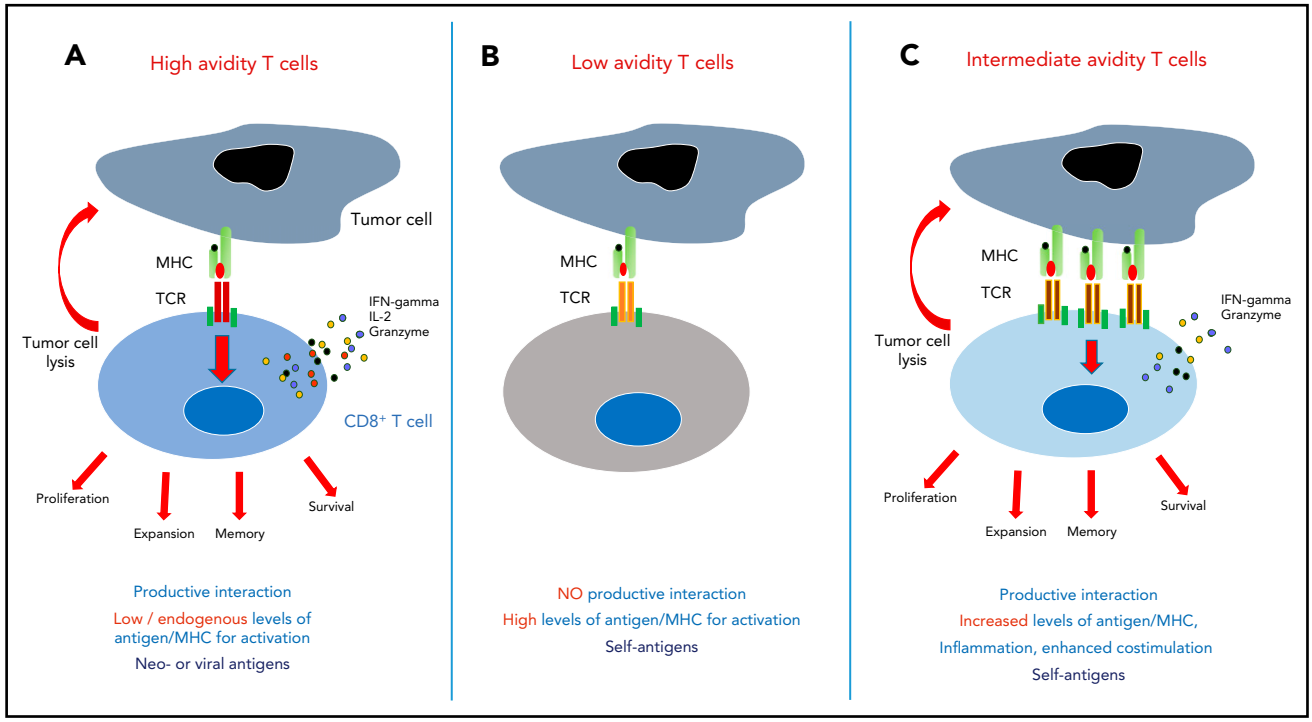
In this issue of *Blood*, Roex et al analyzed the frequency and functional capabilities of self-restricted tumor-associated antigen (TAA) specific T-cell clones isolated from peripheral blood of healthy donors.[1] They found that high-avidity T cells were not detectable, whereas a very limited number of intermediate avidity T cells, with the ability to recognize malignant cells, could be identified. In addition, most of the analyzed clones were nonfunctional and of low avidity, suggesting that a strong and meaningful immune response has to be elicited in cancer patients using a combination of complementary treatment options designed to increase T-cell responses and to enhance antitumor activity of vaccinations containing such TAAs.[2]

Most TAAs that were identified and used in immunotherapeutic approaches represent nonmutated self-antigens that are preferentially expressed or overexpressed in malignant cells.[3] Accordingly, epitopes from these TAAs are recognized by T lymphocytes with low or intermediate affinity T-cell receptors (TCRs) that require high levels of antigen to become activated and often fail to eliminate cells endogenously expressing the cognate antigen.[3,4] However, based on preclinical studies and results from vaccination trials, it was observed that T cells generated against these TAAs are able to recognize malignant cells and contribute to tumor regression. In addition, infusion of isolated tumor infiltrating lymphocytes as well as the adoptive transfer of ex vivo-generated TAA-specific T lymphocytes were shown to induce clinical responses in some patients with relapsed/refractory diseases, demonstrating that, under certain conditions, self-reacting T cells can mediate tumor cell elimination while sparing their nonmalignant counterparts.[5,6]

T lymphocytes recognize via the TCRs the cognate antigens that are presented as short antigenic peptides in the groove of the major histocompatibility complex on the surface of infected cells, malignant cells or antigen-presenting cells (APCs). T cells bearing receptors that strongly react with self-peptides are removed in the thymus (negative selection), referred to as central tolerance.[7] A key mechanism that mediates the elimination of autoreactive cells from the developing polyclonal T-cell repertoire is the ectopic expression of tissue-restricted antigens by medullary thymic epithelial cells. Deletion of most aggressive T lymphocytes is also accomplished by immature dendritic cells or stromal cells in the lymph nodes that present the self-antigens. In addition, there is convincing evidence that regulatory T cells play a crucial role in maintaining tolerance.

Thus, thymic and peripheral tolerance ensure that high-avidity autoreactive T cells are negatively selected and deleted, but both spare cells that weekly recognize antigenic peptides. Low-avidity T lymphocytes can enter the periphery and persist without losing their effector functions, indicating that the threshold for their activation in the periphery is below of that required for negative selection. A weak peptide antigen that failed to delete self-reacting T lymphocytes can induce T-cell activation in the periphery because of higher ligand density, inflammation, or presentation by activated professional APCs (see figure).[8]

Roex et al analyzed the escape of TAA-specific T cells from negative selection and the avidity of these T cells by functional characterization of T-cell clones isolated from peripheral blood of healthy individuals. To accomplish this, they first used as a model minor antigen (MiHA) specific T cells that recognize the HLA-A2 binding HA-1H peptide. MiHA are peptides derived from polymorphic genes and can be recognized as foreign in the setting of allogeneic transplantation when there is a disparity between the donor and recipient. MiHA-reactive T cells were shown to mediate the elimination of leukemic cells and to induce the graft-versus-host disease. High-avidity T-cell clones could be isolated wonly from HA-1H$^-$ patients where the used peptide antigen represented a



Avidity and activation of TAA-specific T cells. (A) High-avidity CD8+ T cells that recognize target cells expressing endogenous levels of antigen are directed against non-self epitopes such as viral antigens or neo-antigens. Autoreactive T cells bearing TCRs that strongly react with self-peptides are deleted in the thymus (negative selection) or in the periphery. (B) Low-avidity self-reacting T cells can enter the periphery and need high ligand density to become activated. (C) However, intermediate-avidity T cells recognizing self-restricted TAAs can bypass negative selection in the thymus and mediate tumor cell lysis that depends on increased antigen expression, compared with non-malignant cells, or be promoted by inflammation, enhanced costimulation, or presentation by activated APCs. IFN, interferon; IL, interleukin; MHC, major histocompatibility complex.

foreign antigen. In contrast, in HA-1H+ individuals (self-antigen situation), only 1 clone showed limited cytotoxic reactivity, demonstrating that a very low number of autoreactive functional T-cell clones with intermediate avidity can escape from the thymic and peripheral tolerance.

In the next set of experiments, T-cell clones specific for several HLA-A2 binding epitopes derived from WT1, PRAME, RHAMM, proteinase, and NY-ESO-1 were isolated from the peripheral blood of healthy individuals. In line with the results using the HA-1H peptide, no high-avidity TAA-specific T cells were identified, and only a small number of intermediate avidity clones was isolated. Of 663 T-cell clones directed against the used TAAs, only 3 PRAME- and 1 NY-ESO-specific T-cell clone secreted interferon-γ or were able to lyse HLA-A2+ malignant cells in response to endogenous levels of antigen.

Importantly, similar to previous reports, the authors found that tetramer-positive T cells comprise many cells that are unable to recognize tumor cells and a large number of nonfunctional low-avidity T-cell clones specifically stained with the corresponding tetramer. Detection of vaccine-induced T cells as a surrogate parameter for efficacy using tetramer staining is broadly used for immunological monitoring and may overestimate the generation of efficient anti-tumor-specific responses, supporting the additional application of functional assays such as ELISpot assays, intracellular interferon-γ staining, or cytotoxic assays.

This study shows that a small proportion of self-reacting T cells can escape from negative selection and only a few TAA-specific T cells can be isolated from healthy donors with the ability to recognize malignant cells in an antigen-specific and HLA-restricted manner. This is probably different in cancer patients where the frequency of tumor-specific T cells might be higher (especially in the tumor microenvironment) and T cells might have different phenotypes and effector functions because of chronic exposure to tumor-derived antigens and the immunological composition of the tumor microenvironment. Most of these T cells have an exhausted phenotype characterized by the expression of check point molecules PD-1, LAG-3, and TIM-3. Some of these T lymphocytes can be invigorated by the application of check point inhibitors and mediate tumor regression.[9] Combination of vaccination therapies with strong adjuvants and compounds that stimulate T-cell priming and activation such as check point inhibitors could enhance the induction of anti-tumor-mediated immunity and therapeutic efficacy.[2]

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RED CELLS, IRON, AND ERYTHROPOIESIS

Comment on Berentsen et al, page 480

Do temperature and time matter in cold agglutinin disease?

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In this issue of *Blood*, Berentsen and coworkers describe the clinical features of the largest data set (N = 232) of patients with confirmed cold agglutinin disease (CAD) from 24 centers in 5 countries, suggesting a climate dependence for prevalence and incidence.[1] Furthermore, the long-term follow-up of outcomes of rituximab-based chemotherapy regimens, especially in combination with bendamustine, reveals greater and longer-lasting responses that became deeper over time.

In 1952, Schubothe coined the term "cold agglutinin disease" to distinguish the disorder from cold agglutinin syndrome (CAS), which was secondary to an underlying infection or malignancy.[2] CAD is a rare form of hemolytic anemia and is challenging in every aspect. The term CAD is quite misleading, because it primarily refers to the physiological properties of the autoantibodies to preferentially bind at cold temperatures, not the clinical presentation of patients as a result of cold temperatures. However, it is characterized by various degrees of chronic hemolysis and even hemolytic crisis, which may be triggered by cold temperatures, as well as by febrile illnesses.[3]

The underlying pathophysiology is well characterized: CAD is a distinct B-cell lymphoproliferative disorder of the bone marrow leading to the production of (clonal) cold agglutinins, typically an immunoglobulin M autoantibody against red blood cell antigens. These antibodies bind to the red cell surface at low temperature, depending on the thermal amplitude, followed by erythrocyte agglutination and activation of the classical complement cascade. This leads to deposition of C3b on affected erythrocytes and initiation of the terminal complement cascade. C3b-coated erythrocytes are phagocytosed by macrophages of the reticuloendothelial system, primarily in the liver.[3]

Berentsen and coworkers report, in their impressive cohort of 232 patients, that mean age at diagnosis was 68 years, with a male/female ratio of 0.56. The mean follow-up time was 8 years from diagnosis (range, 0-32 years). Not unexpectedly, 51.7% had cold-induced symptoms at or before diagnosis. Median hemoglobin (Hb) level was 9.2 g/dL, and 26.7% presented with Hb $<$ 8.0 g/dL. Five-year survival was estimated to be 83%, which was significantly higher than reported previously (61%).[4] A total of 38 primarily arterial thrombotic events (strokes and myocardial infarction that had no association with the severity of anemia) were experienced by 12.9% of patients. Interestingly, Berentsen et al are the first to describe a fourfold greater prevalence (20.5 vs 5.0 cases/million inhabitants) and incidence (1.9 vs 0.48 cases/million inhabitants/y) in Norway compared with Lombardy (Italy), most likely as a result of lower temperatures provoking more symptoms of CAD.

The next challenge in CAD is treatment, when necessary, in addition to general management considerations, like thermal protection. Unlike warm antibody hemolytic anemia, corticosteroids or other immunosuppressants are not active and, therefore, are not recommended. B-cell–directed therapies with rituximab monotherapy or the combination of rituximab with other drugs, such as bendamustine, have become the standard treatment for symptomatic patients with CAD (see figure).[3,5] However, those therapies have obvious limitations, because ≥25% of the patients will not respond. There are also the risk of significant toxicities, potential contraindications, and the length of time for response, which is often weeks or even months.[3] Berentsen and coworkers are able to show, at the latest follow-up of 45 patients with bendamustine-rituximab treatment, that there was a further improvement in response increasing from 71% to 78%, with complete responses increasing from 40% to 53% and a response duration $>$ 88 months.[1,6] This is encouraging and justifies following patients closely after combination chemotherapy and before starting additional, and potentially unnecessary, treatment.

Despite the response to treatment with an increase or even normalization of Hb, patients often continue to hemolyze, as demonstrated by elevated bilirubin and reticulocyte counts with ongoing symptoms of fatigue or other related complications, as a result of complement activation. Unfortunately, patient-related outcomes were not analyzed in this study.

Taken together, these data show the medical need for new, fast-acting, and safe treatments. As shown recently for the anti-C1s antibody sutimlimab in CAD, this complement-directed therapy is able to shut down hemolytic activity almost immediately.[7,8] Therefore, treatment strategies in the future must focus on the foremost problems in CAD (eg, hemolysis, anemia, and acrocyanosis) with the use of suitable regimens and/or combinations of complement-inhibition and B-cell–directed therapies, as reported previously.[3,8]

Once again, this work by Berentsen et al demonstrates the importance of collecting