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■ STRUCTURAL BIOLOGY

Taking Aim at Bioterrorism

Phan J, Lee K, Cherry S, Tropea JE, Burke TR Jr, and Waugh DS. High-resolution structure of the *Yersinia pestis* protein tyrosine phosphatase YopH in complex with a phosphotyrosyl mimetic-containing hexapeptide. *Biochemistry* 42: 13113–21, 2003.

Yersinia pestis, the causative agent of plague, is arguably the deadliest pathogen in history. At least 200 million deaths have been attributed to plague in recorded history. Plague outbreaks are rare in developed countries and can readily be controlled by antibiotics when they occur. However, some recent clinical isolates of *Y. pestis* were reportedly resistant to all of the drugs commonly used for plague prophylaxis and therapy, indicating that the spread of drug resistance may soon become a serious problem.

A more ominous threat, however, is posed by weaponized forms of *Y. pestis*, such as those developed in the Soviet Union during the late 1980s. Reportedly more virulent than wild-type *Y. pestis*, these strains are also reputed to be resistant to all of the antibiotics clinically effective against plague. It would not be surprising if weaponized strains of *Y. pestis* were currently being developed outside the former Soviet Union, in some cases by the same Soviet scientists who were previously engaged in offensive biological weapons research.

Vaccines are clearly one line of defense against weaponized plague, and it is

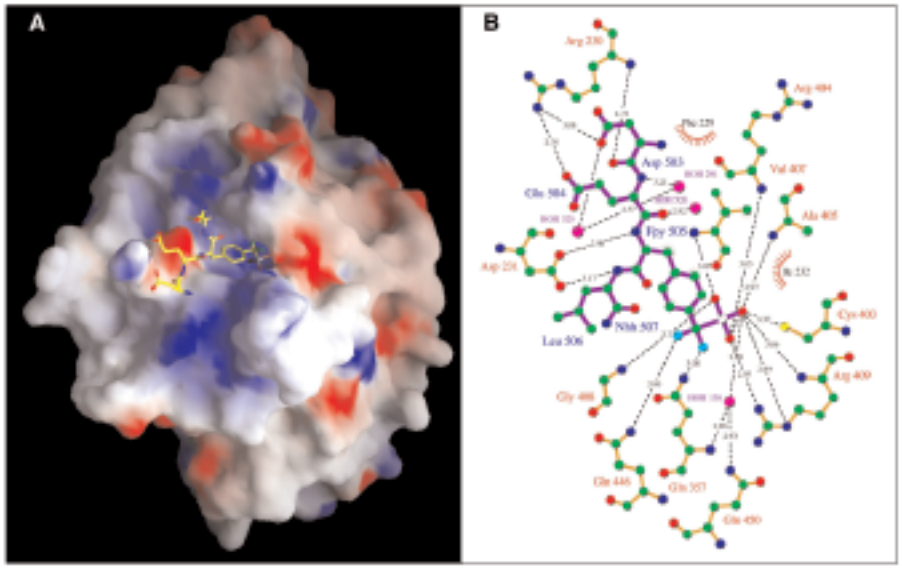

Figure 1. Crystal structure of YopH in complex with a nonhydrolyzable hexapeptide substrate analog. (*A*) Electrostatic potentials mapped onto the molecular surface of YopH with the ligand depicted as a ball-and-stick model. Blue and red represent positively and negatively charged regions, respectively. (*B*) Hydrogen bonding interactions between the enzyme (brown) and the ligand (purple). Carbon, nitrogen, oxygen, sulfur, fluorine, and phosphorus atoms are colored green, blue, red, yellow, light blue, and white, respectively.

reasonable to envision immunization of all military personnel who might be exposed during a conflict. However, vaccination is not a viable strategy to guard against potential civilian casualties of an attack. The current vaccine requires periodic boosters, and repeated exposure results in adverse effects in a considerable number of people who receive it. Besides, the sheer size of the population makes this approach untenable.

Therefore, an antidote for infection is needed—a new kind of drug that cannot be neutralized by genetic engineering or spontaneous mutation as easily as traditional antibiotics. An obvious strategy for developing effective countermeasures against plague is to target the essential virulence factors directly. Because these "molecular terrorists" play a critical role in the pathogenicity of the disease, it may be difficult or even impossible to obtain variants of *Y. pestis* that are resistant to anti-virulence drugs but that can still cause disease. The long-range goal of our research is to facilitate the development of anti-plague drugs via structure-based inhibitor design by attempting to solve the crystal structures of key virulence factors, both alone and in complex with their targets.

The hallmark of *Y. pestis* and many other gram-negative bacterial pathogens is the contact-dependent or type III secretion system (TTSS), which serves to direct the translocation of a small number of cytotoxic proteins, termed effectors, across three membranes from the bacterium into the cytosol of a eukaryotic cell. Collectively, these effectors enable the pathogen to disarm the innate immune response of the infected organism by interfering with crucial signal transduction pathways that regulate actin cytoskeleton dynamics and inflammation. One of the cytotoxic effector proteins that *Y. pestis* injects into mammalian cells via the TTSS is YopH, a potent eukaryotic-like protein tyrosine phosphatase (PTPase). YopH dephosphorylates several proteins associated with the focal adhesion in eukaryotic cells, thereby enabling *Y. pestis* to avoid phagocytosis and destruction by macrophages. Because the PTPase activity of YopH is essential for virulence and the enzyme crystallizes readily, we view it as a particularly promising target for therapeutic intervention.

To establish a framework for the structure-based development of YopH inhibitors, we recently determined the crystal structure of YopH in complex with a nonhydrolyzable hexapeptide substrate analog (Figure 1). The cocrystal structure suggests that the tetrapeptide analog DE-F_2Pmp-L-NH_2, which encompasses the bulk of the interactions with the enzyme active site, would be a good starting point for further optimization. The side chain of the C-terminal leucine residue appears to be a particularly good prospect for modification because it projects into, but does not make optimal contacts with, the body of the enzyme. The crystal structure of the YopH/hexapeptide complex also suggests additional ways in which the inhibitor could be modified to take advantage of adjacent hydrophobic and polar pockets on the surface of the protein.

The challenges that lie ahead are formidable. As in any drug development project, specificity, toxicity, and bioavailability are major concerns. Yet, with the structure of YopH in complex with a nonhydrolyzable substrate analog now in hand, we are potentially one step closer to the eradication of this deadly bioterroristic threat.

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TRANSLATIONAL RESEARCH

A Novel Role for Endothelium-bound SDF-1 Chemokine in Kaposi's Sarcoma Pathogenesis

Yao L, Salvucci O, Cardones AR, Hwang ST, Aoki Y, De La Luz Sierra M, Sajewicz A, Pittaluga S, Yarchoan R, and Tosato G. Selective expression of stromal-derived factor-1 in the capillary vascular endothelium plays a role in Kaposi sarcoma pathogenesis. *Blood* 102: 3900–5, 2003.

Kaposi's sarcoma (KS) is the most common neoplasm in patients with AIDS (Boshoff C et al. *Nat Rev Cancer* 2: 373–82, 2002). The cancer typically involves the extremities and presents as multiple skin lesions containing tumor cells that are referred to as spindle cells because of their microscopic shape. KS is caused by Kaposi's sarcoma–associated herpesvirus (KSHV) (Moore P et al. *N Engl J Med* 332: 1181–5, 1995). A proportion of individuals with HIV infection harbor the virus in a fraction of circulating cells—identified as B lymphocytes and monocytes—and a direct correlation exists between the presence of such cells and the subsequent development of KS. The spindle cells are infected with KSHV, but the surrounding normal tissue is not. Given the predominantly cutaneous location of KS lesions, we investigated how the herpesvirus takes residence in the skin and contributes to the development of this malignancy.

Recently, the expression of selected chemokines and their receptors have been implicated in the recruitment of cells from the bloodstream to specific tissues. Proposed mechanisms involve the generation of chemokine gradients

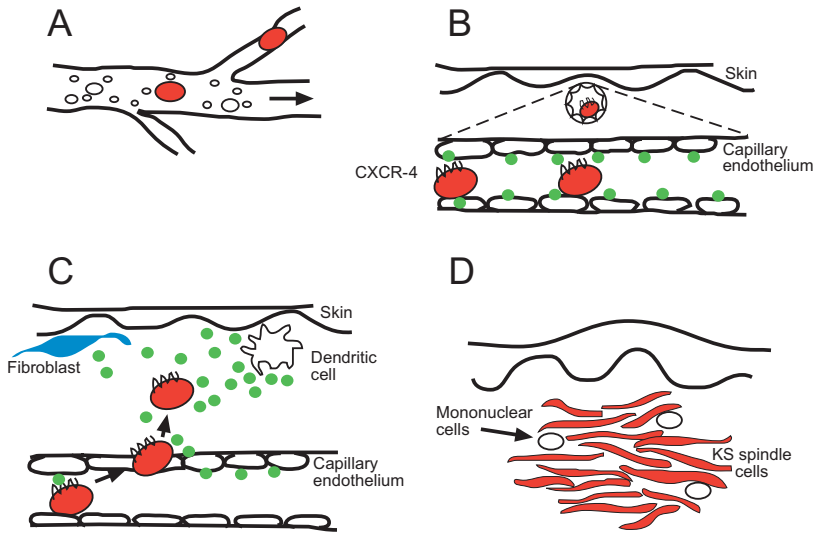

Figure 1. Model for the migration of cells infected with Kaposi's sarcoma–associated herpesvirus (KSHV) to skin tissue. (*A*) KSHV-infected cells (typically B lymphocytes or monocytes), here shown in red, circulate in the blood with other blood cells. (*B*) The infected cells, which express CXCR-4, the receptor for the chemokine SDF-1 (stromal-derived factor-1 [in green]), are arrested by SDF-1–positive capillary endothelium. (*C*) The CXCR-4–expressing, KSHV-infected cells migrate across the capillary endothelium to the extravascular space in the skin in response to an SDF-1 gradient generated by the secretion of SDF-1 by dermal Langerhans' cells and fibroblasts. (*D*) The KSHV-infected cells, now in the skin, contribute to the development of Kaposi's sarcoma.

across the endothelium that would promote transendothelial migration of cells (Campbell JJ et al. *Science* 279: 381–4, 1998). Interestingly, we found that capillary endothelial cells in human skin constitutively express the chemokine SDF-1 (stromal-derived factor-1), whereas most other capillaries do not. Also, human dermal Langerhans' cells and fibroblasts constitutively secrete SDF-1, resulting in the skin having a higher content of SDF-1 than other tissues. Given that most blood cells, including B cells and monocytes, express the receptor for the chemokine SDF-1, namely CXCR-4, we tested the hypothesis that SDF-1 in the skin might serve as a homing signal for KSHV-infected cells.

First, we tested whether endothelial cells expressing SDF-1 can promote the arrest of CXCR-4–positive cells under conditions of flow. In culture, human dermal microvascular endothelial cells express SDF-1 on their surface via attachment to cell surface proteoglycan molecules. Thus, they resemble cutaneous capillary endothelial cells with respect to SDF-1 expression. The KSHV-infected, B-lineage cell line BC-1 and the Burkitt's lymphoma cell line BL-41 express a functional CXCR-4 receptor, whereas the erythroleukemia cell line K562 does not. We found that under conditions that mimic capillary blood flow, BC-1 and BL-41 cells accumulated onto the endothelial monolayer, whereas K562 cells did not. Functional inactivation of CXCR-4, by pretreatment of cells with pertussis toxin, markedly reduced the ability of cells expressing CXCR-4 to accumulate onto endothelial cell monolayers. Stripping SDF-1 from the endothelial cell surface by treatment with specific enzymes (i.e., heparinase I and III), masking cell surface SDF-1 with specific antibodies, and using endothelial cell monolayers that do not express surface SDF-1 virtually abolished BC-1 cell arrest under conditions of flow.

Secondly, we examined whether cells expressing CXCR-4 can migrate across SDF-1–positive endothelium by chemotaxis if a soluble SDF-1 gradient is present across the endothelium. Using transwells separated by a monolayer of SDF-1–positive endothelial cells, we found considerable migration of CXCR-4–expressing cells, in the presence, but not in the absence, of SDF-1 across the endothelial barrier. There was no migration of control cells. Enzymatic removal of surface SDF-1 from the endothelium, or use of an endothelial barrier naturally lacking SDF-1, reduced levels of cell transmigration across the endothelium.

These results show that surface SDF-1 displayed by vascular endothelial cells can promote the specific arrest of cells that express the SDF-1 receptor, CXCR-4. Once arrested, CXCR-4–positive cells can transmigrate across the endothelium in the presence of an SDF-1 gradient (Figure 1). Since the capillary endothelium of human skin expresses SDF-1 and the extravascular skin tissue is rich in SDF-1, these results provide a possible explanation for the preferential recruitment of CXCR-4–expressing/KSHV-infected cells to the skin and the tendency of Kaposi's sarcoma to affect skin tissue. Also, the contribution of SDF-1/CXCR-4 to the pathogenesis of KS suggests that therapeutic manipulations designed to alter SDF-1/CXCR-4 function may prove useful in preventing KS or reducing its dissemination.

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Reporting High-impact Manuscripts

High-impact manuscripts should be reported to Tracy Thompson (thompstr @mail.nih.gov), Chief, CCR Office of Communication, as soon as possible **after acceptance but before publication.** Please include the publication date, an electronic or hard copy of the manuscript, and the journal name. High-impact manuscripts include but **are not limited to** papers that reflect a significant advance in your field or papers in any of the following areas: public health; tobacco-related issues; new technological advances; imaging; obesity, dietary fat, energy balance; nanotechnology; molecular targets; stem cells; angiogenesis; or combination therapies.

Aryl Hydrocarbon Receptor Status Controls Retinoid Homeostasis

Andreola F, Hayhurst GP, Luo G, Ferguson SS, Gonzalez FJ, Goldstein JA, and De Luca LM. Mouse liver CYP2C39 is a novel retinoic acid 4-hydroxylase. Its down-regulation offers a molecular basis for liver retinoid accumulation and fibrosis in aryl hydrocarbon receptor-null mice. *J Biol Chem* 279: 3434–8, 2004.

When in excess, retinoids, compounds in the vitamin A family of molecules, function as chemopreventive agents of epithelial carcinogenesis. Certain carcinogens, however, cause depletion of liver retinoids. Furthermore, liver, intestinal, and other organ tumors appear depleted of retinyl esters, the storage form of vitamin A. Because many carcinogens work by way of binding to the aryl hydrocarbon receptors (AHRs), we hypothesized that AHR deficiency might have an effect on retinoid homeostasis, a parameter normally, and mainly, affected by nutritional vitamin A (retinol) and provitamin A (carotenoid) intake.

The retinoid, retinoic acid (RA), is the ligand of the following nuclear transactivators: retinoic acid receptor (RAR) and (in the 9-*cis*-RA form) retinoid X receptor (RXR). RA is derived from retinol in two steps: first by its transformation to retinaldehyde and then by retinaldehyde's oxidation to RA. Figure 1 shows the relationship between retinol, retinaldehyde (also known as retinal), and RA and indicates the irreversibility of the retinol-to-RA conversion. Thus, once RA is formed, it is used as the ligand of the retinoid receptor families and is catabolized via the formation of 4-hydroxy- and 4-oxo-RA. In the presence of excess RA, catabolic reactions maintain retinoid homeostasis and prevent possible toxicity. Therefore, the importance of the cytochromes as responsible agents of RA oxidation is evident.

We have reported that AHR-null ($AHR^{-/-}$) mice accumulate the three major forms of vitamin A—retinyl esters, retinol, and RA—

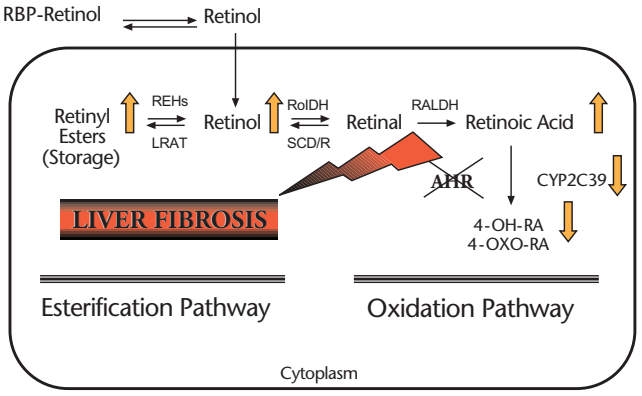

Figure 1. Aryl hydrocarbon receptor (AHR) status is associated with retinoid homeostasis in the mouse liver. The absence of AHR is associated with a downregulation of CYP2C39, a novel retinoic acid 4-hydroxylase, resulting in a disruption of retinoid homeostasis and liver fibrosis. Its presence is associated with the catabolism of retinoic acid to 4-hydroxy- and 4-oxo-retinoic acid, and consequently, the maintenance of retinoid homeostasis and prevention of possible toxicity. RBP, retinol binding protein; REH, retinol ester hydrolase; LRAT, lecithin retinol acyltransferase; RolDH, retinol dehydrogenase; SCD/R, small chain dehydrogenase/reductase; RALDH, retinal (retinaldehyde) dehydrogenase; 4-OH-RA, 4-hydroxy-retinoic acid; 4-OXO-RA, 4-oxo-retinoic acid.

in their livers with a consequent increase in tissue transglutaminase (TGaseII) and transforming growth factor-β (TGF-β) and its receptors. Because retinoids control the transcription of the genes for these proteins, and the increased concentration of these agents coincides with the phenotype of liver fibrosis observed in the $AHR^{-/-}$ mice, we reasoned that nutritional vitamin A depletion would act in the opposite direction, that is, to reduce liver retinoid content and possibly reverse liver fibrosis. Indeed, vitamin A-depleted AHR-null mice showed reduced hepatic stellate cell (HSC) activation and normalized TGaseII levels, thus leading to normal TGF-β activation and signaling, and resulting in normal collagen deposition (Andreola et al. *Hepatology* 39: 157–66, 2004).

Because many carcinogens work by way of binding to the aryl hydrocarbon receptors (AHRs), we hypothesized that AHR deficiency might have an effect on retinoid homeostasis....

In a parallel study, we sought the cause for the retinoid accumulation. Microsomes from the liver of the $AHR^{-/-}$ mice showed a reduced ability to metabolize RA to 4-hydroxy- and 4-oxo-RA, the apparent cause for the accumulation of RA in the liver of $AHR^{-/-}$ mice and, by feedback, for the accumulation of retinol and retinyl esters (Figure 1). To identify the P450 isoform(s) involved in RA metabolism, liver microsomes from AHR-null and wild-type mice were subjected to Western blotting and probed with antibodies to rat P450s that cross-react with murine forms. Signal intensity in Western blots probed with anti-rat CYP2C6 antibodies correlated with levels of RA 4-hydroxylation. Furthermore, the anti-rat CYP2C6 antibodies (1) reduced RA 4-hydroxylase activity of wild-type mouse liver microsomes to the levels of AHR-null mouse liver and (2) exclusively recognized the murine P450 CYP2C39, when used to screen a mouse liver cDNA expression library. *In vitro* catalytic assays of five recombinant mouse CYP2Cs revealed that only CYP2C39 was competent for RA 4-hydroxylation. Real-time reverse-transcriptase PCR used to assess the CYP2C39 mRNA expression showed decreased levels (30%) of this transcript in AHR-null liver compared with wild-type liver, consistent with

decreased protein levels observed by Western blot analysis using an antibody to a CYP2C39-specific peptide. These data show that CYP2C39 catalyzes RA catabolism and thus possibly controls RA levels in mouse liver.

The data are consistent with our interpretation that the downregulation of CYP2C39 is responsible for the liver phenotype in the AHR-null mouse. Although it is clear that CYP2C39 represents a key component of the retinoid homeostatic mechanism, it remains to be determined how the AHR controls CYP2C39 levels. A downregulatory transcriptional effect as a consequence of knocking out AHR expression and the consequent decrease in CYP2C39 protein levels are consistent with the decreased RA metabolism in the AHR-null mouse liver.

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FROM THE DIRECTOR'S OFFICE

NIH Director Dr. Elias A. Zerhouni Visits NCI's Intramural Research Program

NIH Director Elias A. Zerhouni, MD, visited NCI's Intramural Research Program (IRP) in April to learn more about the science being performed by IRP investigators. Encompassing the CCR and the Division of Cancer Epidemiology and Genetics (DCEG), NCI's IRP is a distinctive and highly productive center of basic, translational, and clinical research. Due principally to its ability to make long-term funding commitments and to support high-risk research, the IRP plays a leadership role in advancing cancer research. Partnerships bring together investigators with diverse backgrounds, knowledge, and skills to more rapidly advance scientific discovery both within and outside the NCI. With their partners, the IRP investigators are conducting studies that cannot be easily performed in other settings —studies that are making substantial inroads in the treatment, prevention, and management of cancer and other diseases. Following introductions of the CCR and DCEG, several examples of the high-impact research being performed by IRP investigators were presented to Dr. Zerhouni and are described below.

CCR Director Dr. J. Carl Barrett (*left*) welcomes Dr. Zerhouni to the NCI IRP.

Epigenetic Control of the Genome

The epigenetic code can be thought of as a secondary code superimposed on the DNA code. It is marked by the modification of DNA and its supporting proteins, particularly histone proteins, by such chemical entities as methyl, acetyl, and phosphoryl groups. The epigenetic code, both DNA and protein based, controls gene expression without modifying actual DNA sequences. One of the most important challenges in research today is understanding the full nature of the epigenetic code, in particular, its regulatory mechanisms and how those mechanisms can be exploited to hinder or block completely the molecular routes of cancer.

During the meeting with Dr. Zerhouni, Shiv Grewal, PhD, of the CCR Laboratory of Molecular Cell Biology, discussed his studies of histone modifications. Research from his laboratory has demonstrated that the modification of histones (again by such processes as methylation and acetylation) can change chromatin structure, making it easier or more difficult for genes to be activated. Dr. Grewal also presented evidence that a small RNA–based regulatory network (termed RNA interference or RNAi) plays an important role in epigenetic programming of the genome. Dr. Grewal is striving to define the key molecular events relating to histone modification and RNAi in epigenetic control of gene expression, which very likely will be essential for understanding the mechanisms of carcinogenesis. Dr. Grewal noted that, unlike genetic changes that cause cancer, epigenetic changes that lead to this disease can, in principle, be reversed.

Following the presentation, Dr. Zerhouni asked Dr. Grewal to describe for him how small RNAs promote the targeting of chromatin-modifying activities. Dr. Grewal explained that this may occur in one of two ways: small RNAs could base pair with DNA directly, or base pair with nascent mRNA transcripts that target chromatin modifying activities. In response to Dr. Zerhouni's question of whether or not there are any roadblocks to his research, Dr. Grewal explained that because the epigenetic code is different for different cell types, we need to improve technologies to map histone

modifications or develop epigenetic maps for the various cell types. He went on to say that we could, for example, follow stem cells as they differentiate and monitor for the epigenetic codes of the different cell types as they emerge. There are limits to the current method of mapping histone modifications, termed "ChIP-on-chip," particularly in higher eukaryotic organisms.

Immunotherapy in the Treatment of Cancer

Steven Rosenberg, MD, PhD, of the CCR Surgery Branch, discussed the hallmarks of the translational studies conducted in the Surgery Branch using immunotherapy to treat human cancer. He discussed work leading to the use of interleukin-2 (IL-2) as a treatment for patients with metastatic melanoma and metastatic kidney cancer. IL-2 has been approved by the U.S. Food and Drug Administration and is now being used around the world as the most effective treatment for patients with these diseases. It represents the first successful inmmunotherapy for patients with cancer.

Dr. Rosenberg also discussed how research from the Surgery Branch led to the discovery of immune lymphocytes that are capable of recognizing cancer antigens and to the development of cell transfer therapies that involve the adoptive transfer of *in vitro* generated lymphocytes with anti-tumor reactivity into patients with metastatic disease. Recent improvements in cell transfer therapies in the Surgery Branch following lymphodepleting chemotherapy have led to the most effective cancer immunotherapies in humans to date. Approximately 50% of patients with metastatic melanoma receiving this treatment undergo objective cancer regressions.

When asked about obstacles to his research by Dr. Zerhouni, Dr. Rosenberg discussed two principal problems. One is the limited availability of Good Manufacturing Practices (GMP)—quality reagents in the intramural program, which are needed to help translate research findings into clinical practice. "Too often," he said, "We have to rely on pharmaceutical companies to provide such reagents."

Drs. Zerhouni and Gottesman listen to Dr. Rosenberg discuss his work in using immunotherapy to treat human cancer.

The second point he raised is that the current regulatory restrictions for the application of new treatments are too extreme for patients with advanced disease. That is, the restrictions do not take into account the risk-benefit ratio of the application of new therapies to patients whose mortality risk is extremely high. The same restrictions apply to providing new therapies to these patients as providing a new antacid to patients with reflux disease. Dr. Zerhouni was pleased to learn that Dr. Rosenberg is on the committee working to resolve this problem.

The Neuro-Oncology Branch Drug Pipeline

To accomplish the goal of developing effective therapeutic strategies for the treatment of primary brain tumors, Howard Fine, MD, and his colleagues at CCR's Neuro-Oncology Branch (NOB) created a preclinical and clinical drug development pipeline for the identification and evaluation of new therapeutic agents. Within this pipeline, early phase/phase I clinical trials for promising agents are conducted at the NIH Clinical Center. Agents that continue to look promising are then moved forward to either expanded phase II trials at the Clinical Center or through one of the three NCI-sponsored extramural early phase brain tumor consortia. Agents that look promising at this stage are then brought to the large NCI-sponsored extramural cooperative clinical trials groups for definitive phase III evaluation. The pipeline has proven so successful that the NOB has been able to partner with significant numbers of academic investigators and biotechnology/pharmaceutical companies to develop a large number of agents for the treatment of primary brain tumors.

Dr. Fine also discussed how the NOB is building several core resources for the advancement of basic/translational brain tumor research. The most ambitious of these is the Glioma Molecular Diagnostic Initiative (GMDI), a national study whereby tumors from patients with gliomas are molecularly and genetically characterized and prospective corollary clinical data are accrued.

Dr. Zerhouni asked Dr. Fine if he could provide an example of a scientific advance that emerged from this research. Dr. Fine replied, "Through GMDI, we have identified new molecular targets for a subset of malignant gliomas. This has led to a series of national clinical trials with HIDACs, an emerging class of small molecule anticancer agents."

Human Papillomavirus and Cervical Cancer Research

Douglas Lowy, MD (CCR Laboratory of Cellular Oncology) and Allan Hildesheim, PhD (DCEG Hormonal and Reproductive Epidemiology Branch) discussed the various partnerships in which they and colleague Diane Solomon, MD (NCI Division of Cancer Prevention) are involved. They have been studying the natural history of genital human papillomavirus (HPV) infection, its relationship to cervical dysplasia and cervical cancer, and prevention of cervical cancer through improved cervical cancer screening methods and vaccine development. HPV causes cervical cancer by chronic infection of the cervix, which over time may lead to progressive changes in infected cervical cells. Some of their research partnerships, comprising NCI intramural and extramural scientists, have led to the finding that HPV testing is an effective management strategy for women in the United States who have equivocal Pap test results. This finding formed the basis for the development of Consensus Guidelines by professional societies that have significantly changed clinical management of cervical cancer

screening for millions of women in the United States. Furthermore, their laboratory-based research has led directly to the development of the current candidate prophylactic HPV vaccine. Preliminary test results indicate that this vaccine may be safe and effective in protecting against new infection by the HPV types that are targeted by the vaccine. The NCI investigators are now independently testing a version of the vaccine developed by Glaxo-Smith-Kline in a large efficacy trial involving 15,000 women in Costa Rica, where cervical cancer is the most common female cancer. If successful, this trial, in conjunction with pharmaceutical company–sponsored trials, would lead to commercial vaccines with the potential for reducing up to 70% of cervical cancer. The impact on public health would be considerable, as cervical cancer is the second most common cause of cancer deaths among women worldwide.

Curious as to how HPV triggers cervical cancer, Dr. Zerhouni asked Dr. Lowy if he could further explain this process. Dr. Lowy stated that the virus encodes oncoproteins, especially E6 and E7, that deregulate the normal growth regulation of the cell, in part by their ability to inactivate two key tumor suppressor pathways, the pRb and p53 pathways. This leads to a chromosomal imbalance, changes in cellular genes that cooperate with the abnormalities induced by the virus, integration of the viral DNA into host chromosomal DNA, and with chronic infection of the cervix by the virus, progression to severe cervical dysplasia and cancer. The cancer cell continues to express E6 and E7, and their continued expression is critical to the cancer phenotype.

The InterLymph Consortium

During the meeting, Nathaniel Rothman, MD, MPH, MHS of the DCEG Occupational and Environmental Epidemiology Branch discussed his work with InterLymph, an international consortium of non-Hodgkin's lymphoma (NHL) case-control studies. The goal of InterLymph is to confirm and enhance our understanding of the etiology of NHL, the incidence of which has risen worldwide during the second half of the twentieth century. InterLymph is a consortium of multidisciplinary molecular epidemiologic studies conducted in the United States and around the world. Each team of the consortium has selected a distinct area to investigate in depth. The NCI study for example, focuses on environmental samples and serum measures of various occupational and environmental exposures to accompany a detailed history of residence. Dr. Rothman and an international team of investigators from InterLymph have combined the genetic analyses of eight studies and uncovered the first evidence that genetic variation in a key checkpoint gene in the inflammatory response may play an important role in the etiology of NHL. Moreover, each study within InterLymph found at least one statistically significant association that was not replicated when data from all studies were pooled together, strongly supporting the scientific benefit of such consortial efforts as InterLymph.

When asked by Dr. Zerhouni why they were drawn to study NHL, Dr. Joseph Fraumeni (DCEG Director) replied that it was mainly due to the rising incidence of NHL in the general population, and the urgent need to search for the underlying causes of this trend. Additionally, Dr. Zerhouni asked the following: "Considering the overall size and scope of the consortium, how good are your methodologies in measuring environmental exposures across large cohorts and multiple studies?" Dr. Rothman replied that they take a multidisciplinary approach to measuring environmental exposures. For example, they ask subjects where they have lived, use Global Positioning System (GPS) technology to identify exactly where those homes are, use Geographic Information System (GIS) technology to superimpose information on environmental conditions on those geographic areas, and actually go out and take samples from such things as water sources and carpets. Dr. Rothman stated that they are confident that their results are accurate but are always working to improve their methods.

The IRP on the Leading Edge of Research

The IRP is uniquely positioned to play a leadership role in clinical, translational, and basic research enterprises. Dr. Zerhouni was impressed with the research and other information presented to him, referring to the IRP as a "jewel." At the conclusion of the meeting, he strongly encouraged the IRP staff to continue their efforts to do high-risk, high-impact research that is difficult for others to do and to make discoveries at a rapid pace, noting that we must try our best to avoid being in a "me too" or secondary position when it comes to discovery. Research in the IRP is thriving, and we were grateful for the opportunity to show some of our results to Dr. Zerhouni and to have a scientific discussion with him.

■ **J. Carl Barrett, PhD**
Director

Special thanks to L. Michelle Bennett, PhD; Shiv Grewal, PhD; Steven A. Rosenberg, MD, PhD; Howard Fine, MD; Douglas Lowy, MD; Diane Solomon, MD; Allan Hildesheim, PhD; Joseph F. Fraumeni, Jr., MD; Nathaniel Rothman, MD, MPH, MHS; and Patricia Hartge, ScD

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Molecular and Morphological Mitochondrial Compromise in Infants Born to HIV-1–Infected Mothers Receiving Nucleoside Reverse Transcriptase Inhibitor Therapy During Pregnancy

Divi RL, Walker VE, Wade NA, Nagashima K, Seilkop SK, Adams ME, Nesel CJ, O'Neill JP, Abrams EJ, and Poirier MC. Mitochondrial damage and DNA depletion in cord blood and umbilical cord from infants exposed *in utero* to Combivir. *AIDS* 18: 1013–21, 2004.

Perinatal transmission is the primary mode by which HIV-1 is transferred to children, and pediatric HIV-1 infection is a major cause of childhood morbidity and mortality worldwide. Prevention of maternal-fetal HIV-1 transmission has been feasible since 1994, when administration of the nucleoside reverse transcriptase inhibitor (NRTI) zidovudine (3′-azido-3′-deoxythymidine, AZT) during pregnancy was found to inhibit viral transmission by approximately 75%. The NRTI combination of AZT plus lamivudine ([-]-β-L-2′,3′-dideoxy-3′-thiacytidine, 3TC) marketed as a single drug, Combivir, is frequently used in human pregnancy and is a more effective inhibitor of perinatal HIV-1 transmission than is AZT alone (2003 U.S. Public Health Service Task Force Recommendations, available online at http://www.aidsinfo.nih.gov/guidelines/perinatal/PER_062304.html).

AZT and 3TC are "dideoxy-type" antiretroviral nucleoside analogs—NRTIs that are similar in structure to DNA bases. In most viral and mammalian environments, these drugs are mono-, di-, and tri-phosphorylated before becoming incorporated into both nuclear and mitochondrial DNA (mtDNA). Once incorporated, NRTIs not removed by DNA repair terminate DNA chain extension and replication.

In adults with HIV-1 infection, long-term NRTI use has been associated with mitochondrial dysfunction, assumed to be caused by truncation of host mtDNA and inhibition of the mitochondrial polymerase γ. Although most uninfected infants born to women with HIV-1 infection show no clinical evidence of mitochondrial compromise (Perniatal Safety Review Working Group, *J Acquir Immune Defic Syndr* 25: 261–8, 2000), mitochondrial dysfunction and death have been reported in a few children born to women receiving AZT alone or Combivir (Blanche S et al. *Lancet* 354: 1084–9, 1999). We therefore hypothesized that some exposed infants may have sustained molecular and morphological mitochondrial damage in the absence of clinical manifestations.

In this pilot study, mitochondrial morphological integrity was examined by electron microscopy (EM) and mtDNA quantity determined by PCR-chemiluminescence immunoassay detection (PCR-CID). Umbilical cord and cord blood were obtained from uninfected infants born to either women with HIV-1 infection who received Combivir therapy (n = 10) during pregnancy or HIV-1–uninfected women (n = 9).

For the EM analysis of each infant, approximately 10 coded photos of umbilical cord artery cells were examined by a panel of pathologists, and the degree of mitochondrial damage was designated as within normal limits (WNL) or as having (+), (++), or (+++) pathology. Moderate to severe (++ or +++) mitochondrial morphological damage was observed in the umbilical cords of 6 of 9 infants born to mothers with HIV-1 infection who were receiving Combivir during pregnancy, whereas none of 7 unexposed infants showed similar damage (Figure 1). When the samples were decoded, the exposed and unexposed groups showed statistically significant differences ($P = 0.01$). In addition, when the duration of Combivir therapy during pregnancy was examined as a function of mitochondrial EM damage, the correlation was significant ($r = 0.84$, $P \leq 0.001$), suggesting that there may be a causal relationship between maternal Combivir dosing and fetal mitochondrial damage.

The PCR-CID used to quantify mtDNA compares the PCR products obtained when amplifying with primers for a housekeeping gene, the *18S RNA*, and a mitochondrial gene, the *D loop*. The data are expressed as ratios of mtDNA

■ ADMINISTRATIVE LINKS

NCI Training Warehouse

The new NCI Training Warehouse is now available at http://camp.nci.nih.gov/owd/trainware.html. This web site includes links to various NIH and other governmental training resources, including the Center for Information Technology (CIT) Computer Training web site, the NIH Work/Life Center, HHS University, and the Foundation for Education in the Sciences (FAES). There are also links to NIH online training modules focusing on equal employment opportunity, technology transfer, ethics, and computer security.

NCI's Overtime Policy

All NCI employees are reminded that "with the exception of emergencies, overtime must be authorized *both* in advance and in writing. In emergencies, employees may be ordered to work overtime without prior approval, provided that approval is documented the next work day." Employees should consult with their timekeepers if they have any questions about overtime approval. For policy and procedures related to overtime, NCI Timekeepers should consult the *DHHS Guide for Timekeeping*, chapter 9, "Premium Pay and Differentials for General Schedule Employees" http://www.psc.gov/hrs/itas/Premium.pdf.

D loop gene copies/nuclear *18S RNA* gene copies. Cord blood values for children born to uninfected mothers and those born to HIV-1–infected mothers receiving Combivir showed ratios of 544 ± 30 (mean ± SE) and 339 ± 45, respectively. Umbilical cord DNA from the same infants gave ratios of 512 ± 48 for unexposed infants and 323 ± 36 for exposed infants. In both tissues, the mtDNA depletion in Combivir-exposed infants was statistically significant ($P = 0.003$ for cord blood DNA and 0.006 for umbilical cord DNA). The mtDNA levels found in Combivir-exposed infants were reduced by approximately 36% compared with the unexposed infants.

The study showed evidence of mitochondrial morphological and molecular damage, in the absence of clinical symptoms, in HIV-1–uninfected Combivir-exposed infants. The mtDNA depletion was similar to that reported in two previous studies (Poirier MC et al. *J Acquir Immune Defic Syndr* 33: 175–83, 2003, and Shiramizu B et al. *J Acquir Immune Defic Syndr* 32: 370–4, 2003). The umbilical cord mitochondrial ultrastructural damage has not previously been reported in infants but has been observed in a primate model.

The use of NRTIs to inhibit maternal-fetal HIV-1 transmission has been one of the great medical success stories of the war on HIV-AIDS, saving the lives of thousands of children yearly in the United States alone. The risk-benefit ratio is clearly in favor of giving these drugs; however, observations that mitochondrial compromise may be present in NRTI-exposed HIV-1–uninfected infants cannot be ignored. Implementation of mitochondrial surveillance on a molecular level may facilitate identification of children at risk for persistent mitochondrial dysfunction, and this study demonstrates that quantitation of cord blood or infant leukocyte mtDNA may be a useful biomarker of mitochondrial compromise.

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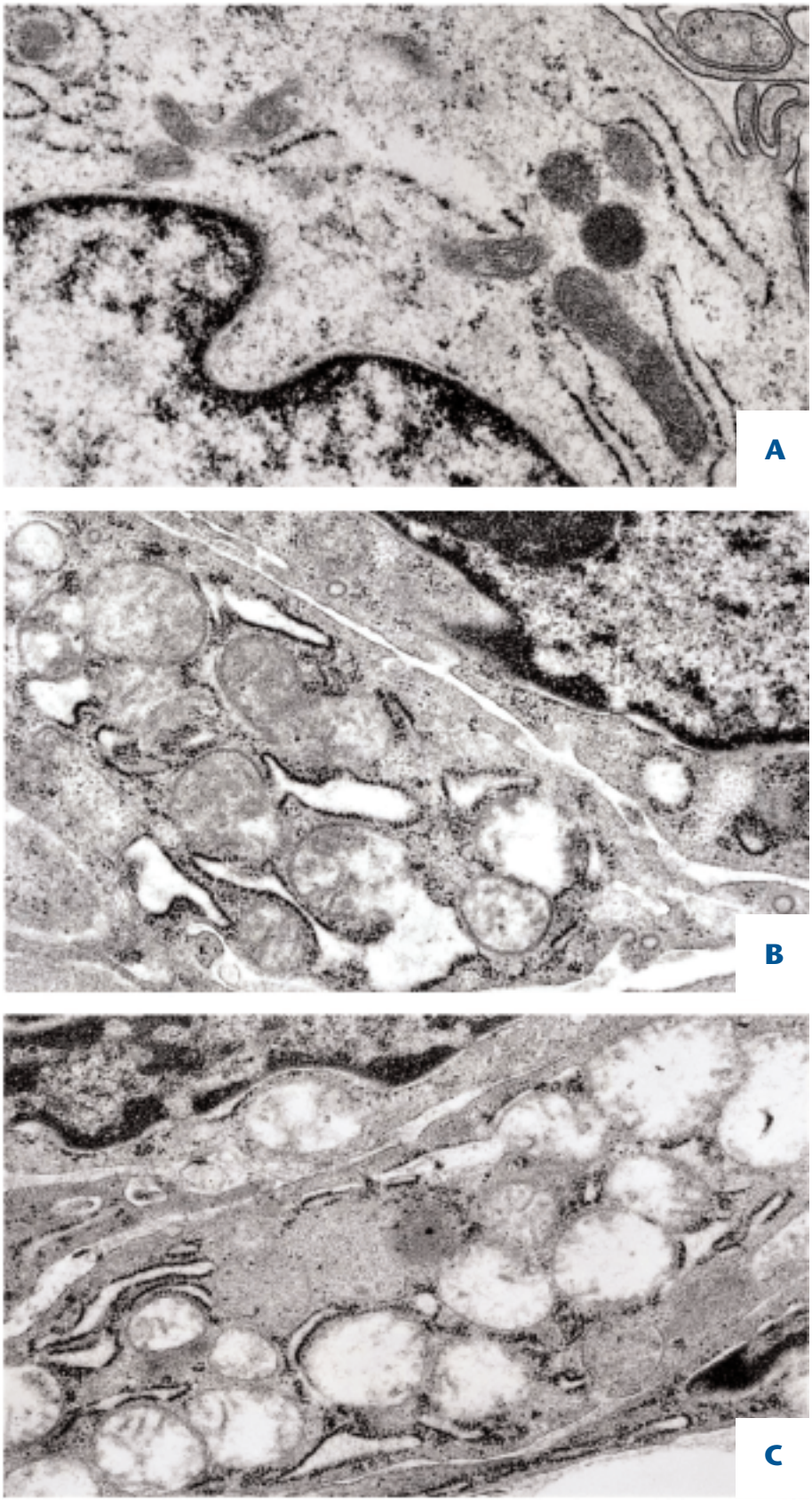

Figure 1. Transmission electron microscopy images of umbilical cord samples from (*A*) an infant born to an HIV-1–uninfected mother with no Combivir exposure and (*B* and *C*) infants born to mothers with HIV-1 infection who received Combivir during their pregnancies. (*B*) shows (++) mitochondrial pathology, and (*C*) shows (+++) mitochondrial pathology.

Transcriptional Regulation by Chaperones and Proteasomes

Stavreva DA, Müller WG, Hager GL, Smith CL, and McNally JG. Rapid glucocorticoid receptor exchange at a promoter is coupled to transcription and regulated by chaperones and proteasomes. *Mol Cell Biol* 24: 2682–97, 2004.

It was a surprise several years ago when we discovered that the glucocorticoid receptor (GR) binds transiently to its promoter target site (McNally JG et al. *Science* 287: 1262–5, 2000). Measurements in live cells revealed that a single receptor binds to the promoter for less than a minute, then falls off to be replaced by a new receptor molecule. A recently developed *in vitro* system has confirmed these observations of transient GR binding at a promoter (Nagaich AK et al. *Mol Cell* 14: 163–74, 2004). Despite this rapid receptor exchange, transcription is upregulated then downregulated on a time course of hours. By using the live-cell binding assay, we sought to answer the following questions: Why is binding and unbinding so rapid, and how is this process regulated?

The live-cell binding assay is performed using fluorescence recovery after photobleaching (FRAP). In this method, a confocal microscope is configured to photobleach a specific spot in a cell. The photobleach is permanent, so the recovery of fluorescence in the spot can only occur by diffusion of unbleached molecules into the spot, and subsequent binding of these molecules to vacant binding sites there. Vacant sites arise only when previously bound molecules are released. Thus, the rate of fluorescence recovery in the spot contains information on the residence time of bound molecules at the promoter. Slower FRAP recoveries indicate longer binding, while faster FRAP recoveries indicate shorter binding.

To measure GR binding in live cells, we used a specially engineered cell line developed in the laboratory of Gordon Hager, PhD. These cells contain 200 contiguous repeats of an element consisting of a GR promoter followed by a reporter gene. This tandem array provides a target site large enough to be seen in the light microscope. When GR is made fluorescent by tagging it with green fluorescent protein, cells containing the tandem array exhibit a distinctly bright spot in the nucleus that always colocalizes with transcripts from the reporter genes. This bright spot is therefore the tandem array, enabling GR binding activity to be measured there.

By using the live-cell binding assay, we sought to answer the following questions: Why is binding and unbinding so rapid, and how is this process regulated?

The regulation of binding can be studied in live cells by perturbing components potentially involved in binding and then examining effects on the rate of FRAP recovery. Using this simple strategy, we investigated whether the rapid exchange of GR was energy dependent. Cells were incubated for 1 hour in deoxyglucose to temporarily deplete ATP levels. This led to a slower FRAP recovery at the tandem array but not elsewhere in the nucleus. Specificity at the array indicates that GR exchange is especially sensitive to ATP levels there. This also illustrates that the exchange process does not reflect a simple chemical equilibrium, but rather is regulated and energy dependent.

There are a number of energy-dependent molecules that might regulate GR binding. We tested two of them—chaperones and proteasomes—and each shows an effect. Inhibition of hsp90 chaperone activity using geldanamycin leads to faster FRAP recoveries at the tandem array (i.e., shorter GR binding times), whereas inhibition of proteasome activity with the proteasome inhibitor MG-132 leads to slower recoveries at the tandem array (i.e., longer GR binding times). Since these effects were detected after relatively short drug treatments (within a few seconds for geldanamycin or 45 minutes for MG-132), they are likely to be specific and direct. In addition, using immunofluorescence, we also detected in normal cells a recruitment of chaperones (hsp90, hsp70, p23) and proteasomes at and around the tandem array site. The simplest explanation for these data is that both chaperones and proteasomes directly affect GR binding at a promoter, with chaperones leading to stabilization and proteasomes leading to destabilization.

Such a balancing act has a precedent in the decision to either refold or degrade a protein in the cytoplasm, a process also mediated by chaperones and proteasomes. This same process, or an analog of it, might be occurring at a GR promoter, but more work is needed to define the precise mechanism. The role of chaperones in particular is complicated by two other recent studies, which have suggested that they may also act oppositely, namely to destabilize GR binding, either at specific promoters (Freeman BC et al. *Science* 296: 2232–5, 2002), or elsewhere in the nucleus (Elbi CC et al. *Proc Natl Acad Sci U S A* 101: 2876–81, 2004).

Regardless of the mechanism, cells evidently expend considerable effort to regulate the rate of GR exchange at a promoter. What does this accomplish? We propose that the rate of GR exchange helps regulate transcriptional levels. This model derives from our observations that slower GR exchange is normally associated with more transcription. We have observed this correlation in seven different cases, where different transcriptional states either arise naturally in cells, or are induced by different hormones or drugs. The simplest explanation at present is that longer GR-residence times at a promoter lead to a better chance that

the transcriptional complex is assembled and, therefore, to more transcription on average. Conversely, faster unbinding may occur when a reduction in the rate of transcription is required.

In summary, our work now suggests that rapid exchange of GR at a promoter is a complex process regulated in some way by chaperones and proteasomes at the least. One function of this exchange may be to tune transcriptional output from a promoter. Live-cell analysis will therefore be critical for a complete understanding of how transcription works.

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■ IMMUNOLOGY

A Novel Multi-component Immunoregulatory Pathway Blocks Tumor Immunosurveillance

Terabe M, Matsui S, Park JM, Mamura M, Noben-Trauth N, Donaldson DD, Chen W, Wahl SM, Ledbetter S, Pratt B, Letterio JJ, Paul WE, and Berzofsky JA. Transforming growth factor-beta production and myeloid cells are an effector mechanism through which CD1d-restricted T cells block cytotoxic T lymphocyte-mediated tumor immunosurveillance: abrogation prevents tumor recurrence. *J Exp Med* 198: 1741–52, 2003.

Although the immune system detects and destroys many foreign organisms and abnormal cells, cancer cells escape this immunosurveillance. Many studies have been conducted to understand their escape mechanisms. Most of the studies, however, have addressed only one element among several that may be involved in sequential mechanistic steps. Uniting many of the elements into one pathway may help us to better understand the escape mechanisms of cancer cells and may provide better insights for designing optimum cancer immunotherapies.

By using a mouse fibrosarcoma model, we have succeeded in connecting some of the elements suppressing cancer-cell immunosurveillance into an immunoregulatory circuit. This tumor cell line shows a growth-regression-recurrence pattern in BALB/c mice that are challenged subcutaneously. We previously showed that tumor regression is mediated by $CD8^+$ cytotoxic T lymphocytes (CTLs), and that downregulation of CTLs by interleukin 13 (IL-13)—but not IL-4—made by $CD4^+$ CD1d-restricted natural killer T (NKT) cells prevented complete tumor elimination. A question remaining in the former study was how IL-13 suppresses CTLs, considering that T cells do not express IL-13 receptors.

We conducted the current study to investigate the identity of cells responding to IL-13 and the mechanism by which they suppress CTLs. To identify the cell population responding to IL-13 to downregulate immunosurveillance, we performed a T-cell transfer experiment by using RAG2 knockout (KO) and RAG2/IL-4Rα double KO mice. Neither mouse strain could reject the subcutaneously injected tumors because they do not have T or B cells. Tumors grew, then regressed, and later recurred in RAG2 KO mice that received T cells transferred from either wild-type mice or from IL-4Rα KO mice. (IL-4Rα is a component of IL-13R.) This result indicated successful reconstitution of RAG2 KO mice with both effector

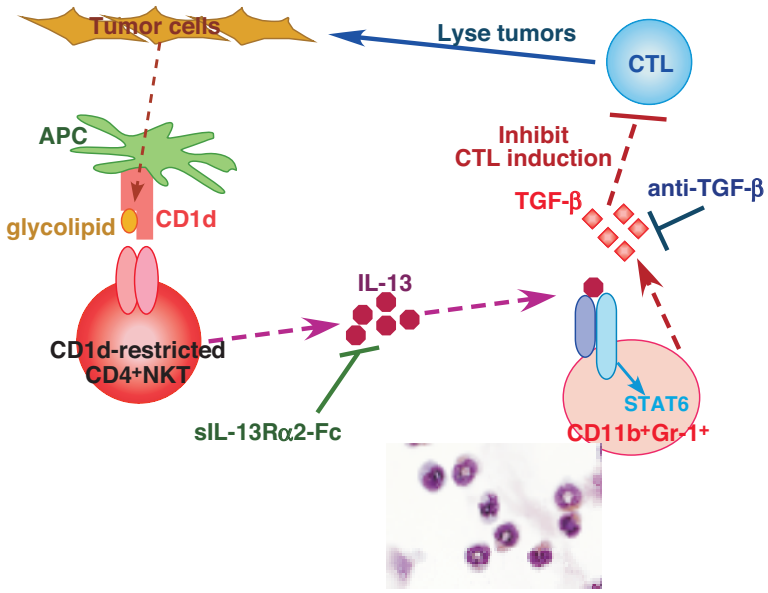

Figure 1. Proposed model of a novel, negative immunoregulatory circuit of cytotoxic T cell (CTL)–mediated tumor immunosurveillance mediated by transforming growth factor-β (TGF-β), myeloid cells, interleukin 13 (IL-13), and CD1d-restricted T cells, probably natural killer T (NKT) cells. Tumor antigen (glycolipid) presented by antigen-presenting cells (APC) via the CD1d molecule is recognized by and activates CD1d-restricted $CD4^+$ NKT cells. The activated $CD4^+$ NKT cell produces IL-13, which acts on $CD11b^+Gr\text{-}1^+$ myeloid cells expressing the IL-13 receptor via the signal transducer STAT6. The $CD11b^+Gr\text{-}1^+$ myeloid cell produces TGF-β to suppress the $CD8^+$ CTLs that can kill tumor cells, thereby downregulating tumor immunosurveillance. This downregulation can be blocked by IL-13 inhibitor (sIL-13Rα2-Fc) or anti–TGF-β antibody, thus preventing tumor recurrence. The insert shows the morphology of the sorted $CD11b^+Gr\text{-}1^+$ cells collected by cytospin and stained with Wright-Giemsa stain. The population contains monocytes and immature granulocytes with "band" morphology. (Adapted with permission from the Rockefeller University Press; Terabe M et al. *J Exp Med* 198: 1741-52, 2003.)

CTLs and regulatory NKT cells. Further, because wild-type T cells and T cells from IL-4Rα KO mice (which cannot react to IL-13) successfully reconstituted RAG2 KO mice, expression of IL-4Rα as a part of the IL-13R on T cells is not necessary for the suppression of immunosurveillance. In contrast, wild-type T-cell–reconstituted RAG2/IL-4Rα double KO mice were protected from tumor recurrence, although transferred T cells expressed IL-4Rα. Therefore, some other cells from the host RAG2 KO recipient mice (that is, cells other than T or B cells) needed to respond to IL-13 to downregulate the CTLs.

To understand the mechanism by which non-T/non-B cells suppress CTLs, we examined their production of the immunosuppressive cytokine, transforming growth factor-β (TGF-β). Splenic nonlymphoid cells (non-T/non-B/non-NK cells) from tumor-challenged mice produced more TGF-β *ex vivo*, without any *in vitro* stimulation. When CTLs were induced *in vitro* in the presence of non-T/non-NK cells from tumor-bearing mice, CTL lytic activity was suppressed. This suppression was completely overcome by adding anti–TGF-β antibody to the culture. Moreover, anti–TGF-β treatment protected mice from tumor recurrence *in vivo*. Therefore, TGF-β made by nonlymphoid cells was necessary for the suppression of immunosurveillance in tumor-bearing mice.

It was still unclear, however, whether TGF-β was induced by IL-13, which has also been shown to be necessary for this negative regulation, or whether both cytokines worked in parallel. To distinguish these two possibilities, we examined *ex vivo* TGF-β production in nonlymphoid cells from mice treated with IL-13 inhibitor after tumor challenge. The cells from IL-13 inhibitor–treated mice produced little TGF-β. Because NKT cells were shown to be a primary source of IL-13 in tumor-bearing mice, we also examined *ex vivo* TGF-β production in nonlymphoid cells from tumor-challenged NKT cell–deficient CD1d KO mice: The cells from CD1d KO mice failed to produce TGF-β. These results indicate that both IL-13 and NKT cells are necessary *in vivo* for TGF-β production by nonlymphoid cells of tumor-bearing mice. By *in vitro* cell depletion experiments, we identified $CD11b^+Gr\text{-}1^+$ cells (a mixture of monocytic cells and granulocytes) as a major source of TGF-β among the nonlymphoid cells of tumor-bearing mice. Finally, the finding that anti–Gr-1 treatment protected mice from tumor recurrence indicated that the $CD11b^+Gr\text{-}1^+$ cells were necessary for the downregulation of tumor immunosurveillance *in vivo*.

We delineated a novel, negative, multicomponent immunoregulatory circuit that blocks tumor immunosurveillance (Figure 1). Tumor growth induces CD1d-restricted NKT cells to make IL-13, which then acts on $CD11b^+Gr\text{-}1^+$ myeloid cells to make TGF-β, which directly suppresses CTL induction. Our study combined previous independent findings regarding TGF-β and myeloid cells together into one pathway. Discovery of this pathway suggests a potential new approach to immunotherapy by synergistically blocking both IL-13 and TGF-β alone or in combination with vaccine or adoptive immunotherapy.

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CCR frontiers IN SCIENCE

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