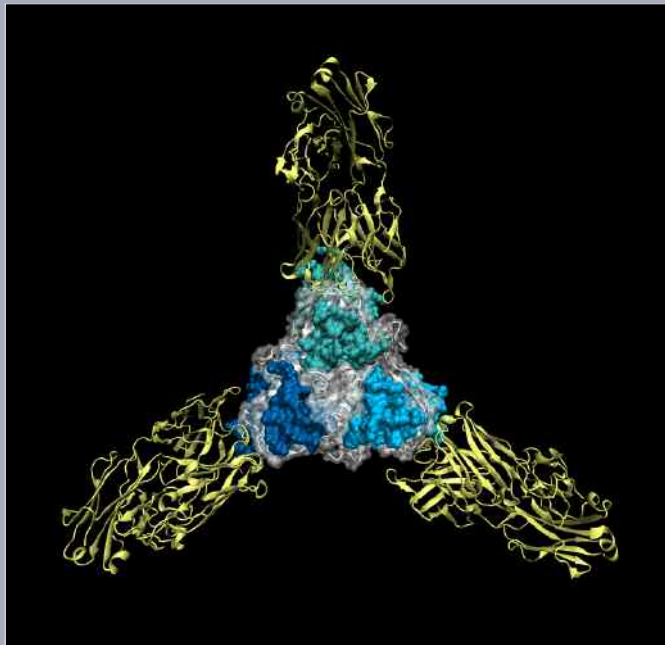


## Immunology and Microbial Science



Crystal structure of the trimeric Ebola virus glycoprotein in complex with a human antibody derived from the serum of a survivor of an Ebola virus outbreak. Artwork by Christina Corbaci, administrative assistant, and Jeff Lee, Ph.D., senior research associate, in the laboratories of Erica Ollmann Saphire, Ph.D., associate professor, and Dennis Burton, Ph.D., professor.



*Dafna Abelson, Research Assistant, and Erica Ollmann Saphire, Ph.D.,  
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*\* Joint appointment in the  
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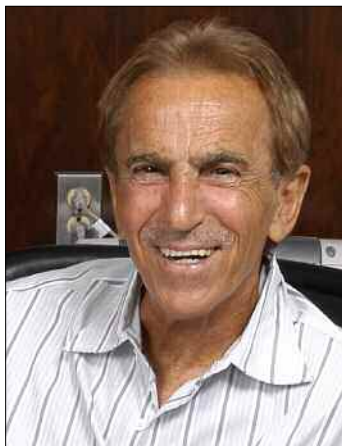
*\*\* Joint appointment in the  
Department of Molecular Biology*

*\*\*\* Appointment completed; new  
location shown*

*\*\*\*\* Appointment completed*

## Chairman's Overview

The Department of Immunology and Microbial Science has seen a great many changes this past year. First was the retirement of Richard Ulevitch from the chairmanship after 14 years of exceptional service. His steady leadership and clear focus allowed the department to thrive even during challenging times. His many seminal scientific contributions in the field of innate immunity have been, and continue to be, widely recognized. In consideration of Dr. Ulevitch's exceptional service, the president of Scripps Research, Richard Lerner, conferred upon him the title of chairman emeritus, and we are happy that he will maintain a presence among us as a professor of immunology. The faculty, as a whole, wishes him all the best in his new endeavors.



Argyrios Theofilopoulos, M.D.

I am indeed honored to have been entrusted by the president with the stewardship of the department, and I thank my colleagues for their advice and assistance.

Dr. Lerner's confidence in the department is clear in the recent initiative to encompass in our faculty several illustrious virologists previously affiliated with various other departments. This merging of highly complementary investigators, technologies, and methods broadens the mission of the department in exciting ways and provides new opportunities to break uncharted scientific ground and to enhance the reputation of Scripps Research as a whole. The Department of Immunology and Microbial Science now has 25 professors, 4 emeritus professors, 15 associate professors, 9 assistant professors, and 132 research associates, staff scientists, and adjuncts.

The caliber of research in the department has long been our strength, as recently exemplified by the election of Michael Oldstone to the National Academy of Sciences, the latest of many well-deserved honors he has received throughout his career. Frank Chisari is also a member of the academy, and we are proud to include 2 members of this highly distinguished group of scientists in our department. We also congratulate Bruce Beutler, a former member of this department and currently chairman of the Department of Genetics, and Peter Wright,

chairman of the Department of Molecular Biology, on their election to the National Academy of Sciences.

On a more somber note, we mourn the passing and celebrate the life and memories of Frank Dixon, a world-renowned immunopathologist, founder of Scripps Research, and first chairman of this department. Dr. Dixon led the way in the study of mechanisms by which viruses and immune complexes result in a broad array of autoimmune and other diseases. Although his highly distinguished achievements and honors are well recognized, it is his role as a mentor, colleague, and friend that resonates most deeply with many of us who were privileged to work with him.

Members of the department continue to publish seminal findings in virology, autoimmunity, cancer, cell biology, and other areas. Although we take great pride in the depth and breadth of the entire body of work of our staff, which is detailed in the ensuing individual reports, a few highlights deserve mention.

Dennis Burton and colleagues published 2 important articles that may have considerable impact on the treatment of AIDS and hepatitis C. In research reported in *Nature*, they found that broadly neutralizing human monoclonal antibodies against HIV require interactions with Fc receptors on effector cells to exert their full protective effects. In other research reported in *Nature Medicine*, they identified human monoclonal antibodies that neutralize genetically diverse isolates of hepatitis C virus (HCV) and protect against challenge with heterologous quasi species of HCV in a human liver-chimeric mouse model. These results raise the hope of using such antibodies to HCV to protect against heterologous viral infections and suggest that a broadly active vaccine against this virus may be possible.

Michael Oldstone and members of his laboratory reported in the *Journal of Experimental Medicine* and earlier in *Nature Medicine* that IL-10 is a major immunosuppressive factor that compromises T-cell antiviral responses; conversely, blockade of IL-10 promotes T-cell responses and enhances clearance of persistent viral infections. These findings have important implications for restoring T-cell activity and creating more efficacious vaccination protocols in many viral diseases, including HIV and hepatitis B and C, in which immunosuppression and vaccine inefficiency are common occurrences.

Philippe Gallay and Frank Chisari published the results of 2 studies in the *Proceedings of the National Academy of Science* in which they identified an amphipathic  $\alpha$ -helical peptide, C5A, that has marked virocidal

effects on both HIV and HCV. C5A corresponds to the N terminus of the membrane-associated nonstructural protein 5A of HCV, an essential component of the viral replication complex. Because of its unique mechanism of action, the development of C5A is an important addition to protease inhibitors currently in use to treat HIV disease and is under development for the treatment of hepatitis C.

Jason Whitmire and Lindsay Whitton published results in the *Journal of Immunology* that challenge current dogma that early inflammation, particularly production of IFN- $\gamma$ , programs contraction of the T-cell population. They clearly showed that instead, IFN- $\gamma$  exerts a pronounced positive effect throughout the T-cell response and that CD4<sup>+</sup> and CD8<sup>+</sup> T cells that cannot respond to this cytokine are 100-fold less likely to enter the memory pool than are CD4<sup>+</sup> and CD8<sup>+</sup> T cells that can respond. Moreover, in research described in *PLoS Pathology*, they showed that both memory and naive T cells have an extended and indistinguishable delay in the onset of proliferation in response to antigen, a finding contrary to the generally held belief that memory T cells initiate division much more rapidly than do their naive counterparts. This delay in the proliferation of memory T cells appears to provide an evolutionary safeguard that balances the risk of infection against the consequence of severe immunopathologic changes.

Erica Ollmann Saphire and colleagues, in a prominent full article in *Nature*, described their success in obtaining the crystal structure of Ebola virus glycoprotein in a trimeric conformation and in complex with a neutralizing antibody fragment derived from a human survivor of a 1995 outbreak of the virus. This major achievement has important implications for understanding the immunopathology of Ebola virus and in advancing efforts to develop vaccines and other therapeutic agents in the event of a natural, accidental, or intentional release of this life-threatening virus. The unique crystal structure obtained was featured on the cover of *Nature*, a signal honor.

Glen Nemerow and colleagues reported in *Cell Host and Microbe* that human  $\alpha$ -defensins inhibit adenovirus infection and that this inhibition requires direct association of the defensin with the virus. Moreover, defensins inhibit virus disassembly at the vertex region, thereby restricting the release of an internal capsid protein required for penetration of the endosomal membrane during cell entry. Thus, defensins have remarkably distinct modes of activity against bacteria and viruses,

and their function may provide insights for the development of new antiviral strategies.

Dwight Kono and colleagues, in research described in *Immunity*, showed that a spontaneous function-impairing mutation of the gene for coronin-1A (an F-actin inhibitor) suppresses lupuslike autoimmunity by reducing T-cell activation and migration. This finding provides a strong rationale for investigating the role of actin-regulatory proteins in autoimmunity and their potential as therapeutic targets.

In an article in *Nature*, Wolfram Ruf, Hugh Rosen, and colleagues described a novel mechanism by which hyperactivation of the immune system can lead to bacterial sepsis. They found that thrombin induces signaling by the protease-activated receptor 1 on dendritic cells, thereby amplifying inflammatory responses that, surprisingly, take place not in the vascular bed but rather in the lymphatic system. They further showed that blocking interactions between this receptor and sphingosine 1-phosphatase receptor 3 can interrupt systemic inflammation and death due to bacterial sepsis in experimental animals, thereby providing a potential new approach for the treatment of this condition in humans.

In research reported in the *Journal of Experimental Medicine*, Linda Curtiss and Peter Tobias made an important connection between atherosclerosis and inflammation by showing that endothelial cells in regions of disturbed blood flow on arterial walls express Toll-like receptor 2 and by providing evidence in experimental animals that interactions of either oxidized lipoproteins or bacteria with this receptor can provoke inflammation and subsequent atherosclerosis. Dianne Mackay also obtained evidence, presented in the *Journal of Immunology*, that Toll-like receptors expressed in kidney allografts play a role in rejection and in ischemia-perfusion injury, suggesting that blockade of these receptors and other molecules associated with innate immunity may be a new approach for the treatment of atherosclerosis and allograft rejection.

Michael G. McHeyzer-Williams and colleagues published an article in *Nature Immunology* on research in which they found that high-affinity follicular B helper T cells are important participants in the induction of humoral responses against protein vaccines. In another article, in *Immunity*, they reported that the nature of the adjuvant used in vaccination greatly influences the clonal composition of the responding T cells. These findings have important implications for the design of effective protein subunit vaccines.

In research described in *Immunity*, David Nemazee found that receptor editing, necessary for B-cell self-tolerance and formation of light chains, is highly dependent on intact recombination sequences. Mice with mutations of the kappa-deleting element are the first mouse strain with a defect solely in autoreactive B-cell editing. Another study by Ann Feeney, published in the *Journal of Experimental Medicine*, indicated that some lupus-prone mice have partial defects in the control of B-cell tolerance mediated by receptor editing.

Gary Bokoch and his group continue to advance our understanding of the Rho GTPase signaling mechanisms that underlie cell division and motility. In articles published in *Developmental Cell* and *Molecular and Cellular Biology*, they reported the crucial role of the Rho regulator guanine nucleotide exchange factor H1 in coupling the polymerization state of microtubules to cell contractility. An example is the key role of the factor in regulating formation of cleavage furrows during cell division. Dr. Bokoch and his coworkers have also shown that Rac GTPase acts through its downstream mediator, cofilin, to regulate the coupling of dynamic actin networks at a cell's leading edge that control motility. These results establish new insights into how cell signals couple to the molecular machinery that regulates actin dynamics and motility, findings particularly relevant to chemotaxis of phagocytic leukocytes.

Reviewing these contributions has deepened my appreciation of the quality of the scientists in the department and has instilled in me a sense of pride and confidence that we will continue to push the cutting edge of biomedical research. As Dr. Lerner recently said, the fact that we continue to list such achievements despite the current economic difficulties speaks volumes about the talents, skills, and innovation of our faculty.

Finally, it is important to acknowledge the integral and crucial functions of our support staff, who are a great credit to Scripps Research. We are fortunate to be able to depend on laboratory, office, and administrative staff who ensure our success in myriad indispensable ways. From the smooth work flow in our laboratories, offices, and technologic service facilities to the administrative departments that keep us abreast of opportunities and ever-changing regulations, the professionalism and dedication of these individuals are valuable for the performance and public dissemination of our science.

In offering my congratulations to my colleagues and coworkers for their achievements, it is my strong belief

that this department will continue to be recognized worldwide as a leader in the study of immunology and microbial biology and that the pioneering contributions of our staff will continue to advance the sciences of medicine and health.



## INVESTIGATORS' REPORTS

# Rho GTPase Regulation of Cell Function in Health and Disease

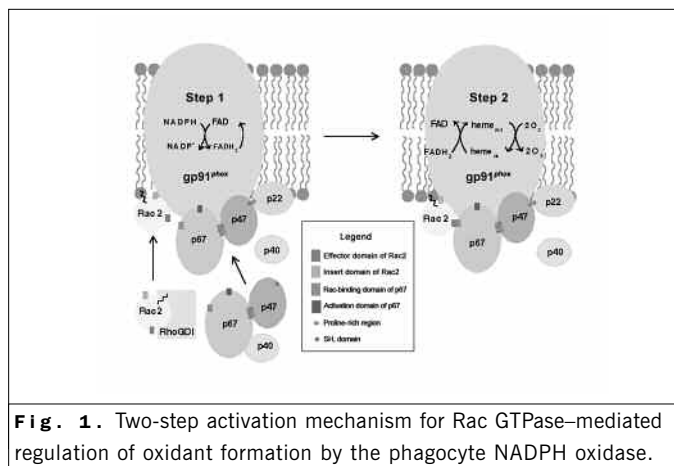
G.M. Bokoch, C. DerMardirossian, A. Anselmo, A. Banerjee, S. Chang,\* V. Delorme, D. Gianni, T. Huang, Y.-Y. Kao, J.-S. Kim, R. Pathak, N. Taulet, H. Zhang, B.P. Bohl, B. Fowler

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**R**ho GTPases control the assembly of the actin and microtubule cytoskeletons, the production of reactive oxygen species, and the activity of kinase cascades that mediate cell growth, death, and motility. This spectrum of activities makes Rho GTPases key components of such physiologic and pathologic processes as tumor growth and metastasis, wound healing, neuronal connectivity, innate immunity, and inflammation. We use cellular, molecular, biophysical, and biochemical approaches to understand how the activities of Rho GTPases are regulated, to identify the proteins the enzymes interact with to control cell function, and to investigate how these regulatory processes are abnormal in various disease states.

### RHO GTPASES AND THE INNATE IMMUNE RESPONSE

We previously established that the GTPase Rac2 regulates the formation of reactive oxygen species used for microbial killing by human phagocytic leukocytes in the innate immune response. Our discovery of a functional interaction between Rac2 and the membrane-bound NADPH oxidase component Nox2 led us to propose a 2-step mechanism for regulating the formation of reactive oxygen species (Fig. 1). We have now mapped the Rac2-binding site on Nox2 as a prelude to investigating the molecular basis for Rac2 action.



**Fig. 1.** Two-step activation mechanism for Rac GTPase-mediated regulation of oxidant formation by the phagocyte NADPH oxidase.

These ongoing studies are providing new insights into NADPH oxidase activation.

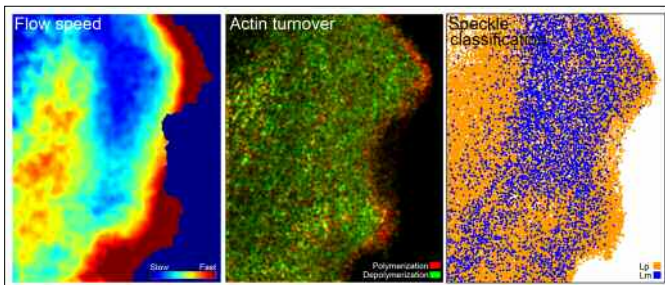
In addition to their role in leukocytes, members of a conserved family of NADPH oxidases (Noxs) are found in other tissues, where the enzymes participate in intracellular signaling. Regulation of nonphagocyte Noxs is largely not understood, but we are investigating the modulation of Noxs by kinase pathways that phosphorylate Nox regulatory components. One pathway for inhibitory regulation of Nox1 in the intestinal epithelium requires the cAMP/protein kinase A-mediated phosphorylation of the regulatory protein NoxA1, which results in binding of 14-3-3 protein. We have shown that this mechanism is induced by the toxins of *Vibrio cholerae* and *Bacillus anthracis* as a means for these bacteria to avoid the innate immune response in the gut. In separate studies, we found that the tyrosine kinase c-Src, long implicated in the development of colon cancer, mediates Nox1 activation through regulation of Rac1 activity. These studies are continuing to provide new insights into the possible contributions of Nox to inflammatory bowel diseases and cancers. A cell-based high-throughput screen for novel Nox regulators is under way.

*Bacillus anthracis* inhibits the function of immune cells by generating lethal toxin and edema toxin. As part of a program grant funded by the Centers for Disease Control and Prevention, we are investigating the molecular basis for the suppressive effects of the anthrax toxins on human leukocyte function and for the roles of Rho GTPases in the uptake and action of anthrax toxins in macrophages.

### CYTOSKELETAL REGULATION BY RHO GTPASES IN CELL MIGRATION AND DIVISION

The p21-activated kinases (PAKs) are Rac and Cdc42 effectors that mediate chemotaxis, wound healing, tumor metastasis, neurite outgrowth, antigen presentation, and other processes dependent on cytoskeletal polarization. In collaborative studies with G. Danuser, Department of Cell Biology, we are using quantitative fluorescent speckle microscopy to investigate the regulation of leading-edge actin dynamics by PAK1 downstream of Rac GTPase. We have found that PAK1 plays an important role in coupling cell-edge protrusion mechanics to upstream signaling events and downstream motility.

The phosphorylation of cofilin, which depolymerizes and severs actin, by PAK1 acting through LIM kinase is an important regulatory point in cell motility (Fig. 2). Using a biochemical screen, we identified a



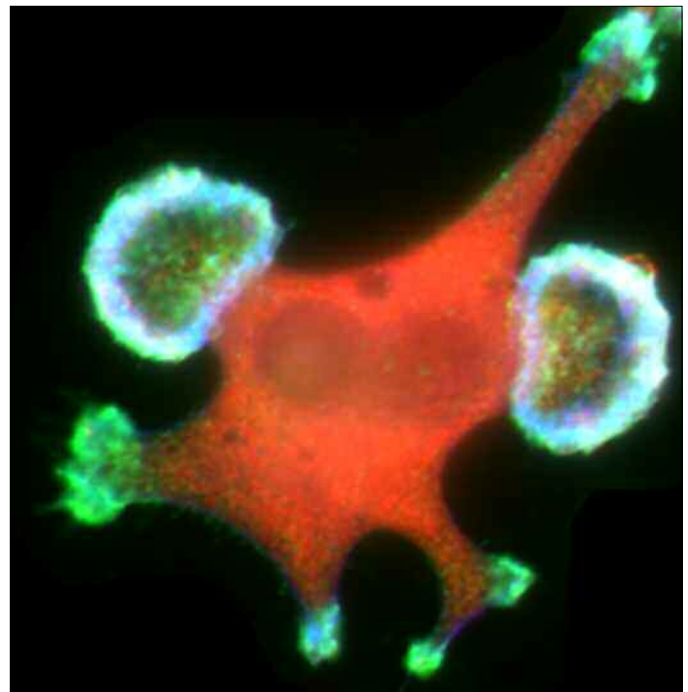
**Fig. 2.** PAK1 regulates leading-edge actin dynamics through cofilin. Images obtained by using quantitative fluorescent speckle microscopy enable the analysis of various parameters characterizing F-actin dynamics, including flow speed, turnover, and classification as the lamellipodia (Lp) or the lamella network (Lm).

unique cofilin phosphatase, termed chronophin, that regulates stimulus-dependent activation of cofilin. We noted roles for chronophin in the control of cytokinesis during cell division, and chronophin has been implicated in the formation of aneuploid cancers. Our recent data indicate that this unique regulatory phosphatase orchestrates actin dynamics at the leading edge by modulating cofilin activity, thereby increasing cancer cell motility stimulated by epidermal growth factor. We have also linked chronophin to a unique mechanism for regulating the formation of rod-shaped inclusions associated with the depletion of cellular energy (ATP) induced by neuronal damage and ischemia. These structures are characteristic of neurodegenerative diseases and may be causally linked to progression of the diseases.

#### GTPASE REGULATORS IN HEALTH AND DISEASE

GDP dissociation inhibitors (GDIs) are critical regulators of Rho GTPase function. They have been linked to kidney disease and to the ability of cancer cells to metastasize. We found that the interaction of GDIs with Rho GTPases is regulated by phosphorylations initiated through various signaling pathways. Indeed, tyrosine phosphorylation may disrupt the regulatory capability of GDIs to promote cell transformation and metastasis (Fig. 3). We are using imaging of live cells to understand quantitatively regulation of the Rho GTPase–Rho GDI cycle.

We have established a mechanism for cross talk between the actin and microtubule cytoskeletons involving Rho regulation via physical sequestration of the Rho guanine nucleotide exchange factor H1 (GEF-H1) by microtubules. GEF-H1 is abundant in blood cells and is downregulated by recently developed drugs that inhibit chronic leukemias. GEF-H1 serves as a link between mitotic spindle microtubules and the initiation of Rho-dependent contractility in a variety of cellular



**Fig. 3.** Src induces the tyrosine phosphorylation of Rho GDI and its location to invasive structures known as podosomes or invadopodia in Src-transformed fibroblasts. Red, F-actin; green, phosphotyrosine; blue, Rho GDI.

processes. GEF-H1 depletion induced by using short interfering RNA has established important roles for GEF-H1 in the regulation of leading-edge cytoskeletal dynamics and motility. We are investigating potential links between these effects of GEF-H1 and vesicle trafficking.

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## Human Antibodies and HIV Vaccine Design

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**H**IV type 1 (HIV-1) is a scourge on humanity. Nearly 40 million people are infected with the virus, and about 20 million have died of AIDS. It is widely recognized that a vaccine is likely the best way to control HIV infection worldwide. All current antiviral vaccines elicit antibody responses that are thought to be crucial to the efficacy of the vaccines. We wish to understand antibody responses to HIV in humans and to design vaccines that will elicit protective responses to the virus.

We used phage display technology to generate panels of human monoclonal antibodies to HIV. We are examining human antibody responses to the virus and the antiviral activities of these antibodies. In particular, we generated a human monoclonal antibody, b12, that neutralizes an array of different strains of HIV. The existence of this antibody indicates that some features of HIV are conserved and are attractive targets for vaccines. Further, b12 and a small number of mono-

clonal antibodies with similar qualities are powerful tools for exploring antibody activity against HIV-1.

Among the first questions we have tackled were the following: Can antibodies protect against HIV-1 infection, and, if so, under what conditions? On the basis of passive transfer studies in a number of animal model systems, the answer is clearly yes. Complete protection is possible at serum titers of neutralizing antibody greater than about 1:100, although lower titers can provide benefit in terms of lowered or delayed viremia. We also showed that topically applied antibody can protect monkeys against vaginal challenge with virus. In addition, the results of passive transfer studies with engineered antibodies in macaques suggest that antibody effector functions, as well as classical neutralization, may be important in protection against HIV.

A major issue is the best method for eliciting protective neutralizing antibodies. Accumulated evidence suggests that protective neutralizing antibodies are those antibodies that bind avidly to the envelope trimer on the surface of HIV-1 virions. However, such antibodies, particularly those to conserved regions of the envelope that are most important for vaccines, are difficult to elicit. Apparently the envelope trimer, which is composed of 2 glycoproteins, gp120 and gp41, has low antigenicity and immunogenicity. Several strategies to circumvent these problems are being investigated.

One strategy is to study the interaction of the neutralizing antibodies with envelope glycoprotein at the molecular level and then use the knowledge gained to design antigens capable of eliciting the relevant antibodies. In these studies, we are collaborating with I.A. Wilson, Department of Molecular Biology. We are also working with P.E. Dawson, Department of Cell Biology, to design peptide immunogens and with C.-H. Wong, Department of Chemistry, and R.A. Dwek, Glycobiology Institute, Oxford, England, to design and select carbohydrate immunogens.

Finally, we are exploring the specificities of antibodies from those rare humans who make antibodies that neutralize a broad array of different strains of HIV. We have evidence that a number of specificities are involved, and we are attempting to describe these. We are using not only phage display but also yeast display and the rescue of memory B cells to generate human monoclonal antibodies.

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## Humoral Immunity to Hepatitis C Virus

D.R. Burton, M. Law, E. Giang

The World Health Organization estimates that 3% of the world's population is infected with hepatitis C virus (HCV). HCV has become a major factor in the incidence of liver cancer, and HCV-associated liver failure is the most common indication for liver transplantation. An effective means to prevent new infections and the reinfection of liver transplant patients is urgently needed.

We are interested in understanding the humoral immune response to HCV. The results of recent studies by groups in France and Germany suggest that the induction of early and potent neutralizing antibody responses to the virus can help control and clear HCV infection. Therefore, it may be possible to protect people at risk for HCV infection by vaccination or in patients undergoing liver transplantation because of HCV infection, to prevent recurrent infection by using neutralizing antibodies. A daunting challenge to a vaccine against HCV is the extreme variability of the virus. The genetic difference between HCV strains can be as much as 30%. For any vaccines or therapeutic antibodies to be effective against HCV, they must be broadly reactive against the many HCV strains circulating in the human population.

In collaboration with colleagues at Scripps Research, at Scripps Memorial Hospital, at the Universities of Nottingham, Birmingham, and Reading in England, and at the University of Alberta in Canada, we have identified several unique human monoclonal antibodies that recognize a relatively conserved antigenic region on the E2 envelope glycoprotein of HCV. We found that the antibodies cross-neutralized many HCV isolates and, most importantly, significantly protected humanized mice challenged with serum from a person with HCV infection. The data indicate that despite the extreme variability of HCV, the virus can be neutralized by targeting a conserved region on the viral envelope glyco-

protein. We are exploring whether this conserved region can be used to design an HCV vaccine and whether these broadly neutralizing monoclonal antibodies can be used as therapeutic antibodies.

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## LABORATORY OF EXPERIMENTAL VIROLOGY

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### Molecular Biology of Hepatitis B and C Viruses and the Immune Response to Their Antigens

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**H**epatitis B and C viruses are noncytopathic DNA and RNA viruses that cause acute and chronic hepatitis and hepatocellular carcinoma. More than 500 million people worldwide are chronically infected, and more than 2 million people die of these infections every year. The focus of our research is to unravel the life cycle of these viruses, discover the roles played by the innate and adaptive immune responses in the control of the infections, and elucidate the mechanisms responsible for viral clearance and disease pathogenesis. Our goal is to devise novel strategies to prevent and cure these infections.

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### Impact of Intrahepatic Antigen Recognition on Priming of the CD8<sup>+</sup> T-Cell Response

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M. Isogawa, F.V. Chisari

**T**he CD8<sup>+</sup> T-cell response contributes to the pathogenesis of liver disease and viral clearance during infection with hepatitis B virus (HBV), and

failure to induce and/or sustain that response results in viral persistence, chronic hepatitis, and hepatocellular carcinoma. To delineate the mechanisms that regulate the CD8<sup>+</sup> T-cell response to HBV, we are using T-cell receptor transgenic mice that have CD8<sup>+</sup> T cells specific for the HBV core and envelope proteins. When adoptively transferred into HBV transgenic mice, naive CD8<sup>+</sup> T cells proliferate vigorously intrahepatically before they appear in lymphoid tissues but do not develop antiviral effector functions, suggesting that intrahepatic T-cell priming induces functionally defective T-cell responses. Intravital imaging suggests that HBV-specific naive T cells are primed by antigen-presenting cells in the hepatic sinusoids of the HBV transgenic mice rather than by the animals' hepatocytes or in peripheral lymphoid organs as widely assumed. These studies provide insight into previously unknown early immunologic events that occur in response to HBV infection. Currently, we are identifying the intrahepatic antigen-presenting cells that prime the T-cell response and the nature of the signal that prevents functional maturation of the T cells.

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### Interaction Between Platelets and Virus-Specific Cytotoxic T Lymphocytes Within the Hepatic Microcirculation

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**U**sing transgenic mice that replicate hepatitis B virus (HBV) at high levels in the liver as recipients of HBV-specific cytotoxic T lymphocytes (CTLs) and normal inbred mice infected with hepatotropic, replication-deficient adenoviruses, we recently showed that platelets play a crucial and previously unrecognized role in viral pathogenesis. Indeed, upon activation, platelets contribute to liver disease and viral clearance by promoting the recruitment of virus-specific CTLs into the liver. As indicated by ex vivo experiments under flow, this remarkable effect most likely depends on specific interactions between platelets and CTLs. Thus, platelet-CTL interactions occurring within the hepatic microcirculation may direct CTLs to extra-

sate, reach parenchymal cells (i.e., hepatocytes), and perform pathogenetic and/or antiviral effector functions.

Intrahepatic visualization and mechanistic understanding of platelet-CTL interactions are major goals of our research program. Through the use of virus-specific CTLs, HBV transgenic mice, and mice genetically deficient in various platelet molecules, we plan to define *in vivo* the molecular basis of platelet-CTL interactions. Through the use of confocal and intravital microscopy, we plan to visualize where such interactions take place within the hepatic venous microvasculature.

The hepatic venous microvasculature consists of postsinusoidal venules and sinusoids, whose hemodynamic conditions, anatomy, and function can vary greatly between different compartments and with time or injury. Furthermore, sinusoidal endothelial cells are morphologically unique, characterized by an absence of tight junctions between cells, a lack of basal membrane, and the presence of open fenestrations. The low-flow sinusoidal environment coupled with the peculiar anatomic and molecular features of this vascular bed may allow leukocyte extravasation through nonconventional adhesion or migration mechanisms (e.g., integrin- or selectin-independent mechanisms). Studying leukocyte extravasation across the hepatic microvasculature in inflammatory conditions may unravel not only new pathways that promote diapedesis but also novel therapeutic approaches for treating chronic hepatitis and other inflammatory diseases.

## Role of Platelets in Controlling Hemorrhage and Effecting Cytotoxic T-Lymphocyte–Dependent Viral Clearance During Lymphocytic Choriomeningitis Virus Infection

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**M**ice infected with lymphocytic choriomeningitis virus (LCMV) have an IFN- $\alpha/\beta$ -dependent platelet dysfunction, and if the level of platelets is less than a critical threshold number, this dys-

function results in severe bleeding and acute anemia and is often followed by death. Moreover, the decreased platelet count and function are linked to a reduction in the virus-specific cytotoxic T-lymphocyte response in blood and infected organs such that viral clearance cannot occur. Platelets therefore play a key role in the progression and severity of LCMV infection in mice, because the cells are essential to control the propagation of the pathogen and the consequences of the pathogen's presence. To perform these functions, platelets require activation; inhibitors of such processes impair the ability of the cells to promote viral clearance and prevent hemorrhage. Although hemorrhage is an expected consequence of reduced platelet function, the effect on a cytotoxic T lymphocyte–dependent process required to block spreading of a virus was previously unknown.

Our results indicate that induction of IFN- $\alpha/\beta$ , with its consequences on platelet number and function, is the key pathogenetic event in this murine model of infection by a member of the Arenaviridae family. Exceptionally high levels of circulating IFN- $\alpha$  are found in humans infected by Junin virus, also an arenavirus, and patients who progress to a fatal outcome often have marked thrombocytopenia associated with platelet dysfunction, mucocutaneous hemorrhage, impaired cellular immunity, and lack of viral clearance. Because fully functional platelets can prevent death in platelet-depleted LCMV-infected mice, even when the number of platelets is less than normal, transfusion of normal platelets should be considered, along with neutralization of IFN- $\alpha/\beta$  activity, in the treatment of life-threatening arenavirus infections in humans.

## Size of the Viral Inoculum and the Kinetics, Quality, and Magnitude of the T-Cell Response and the Outcome of Infection With Hepatitis B Virus

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**W**e previously showed that low-dose viral inocula do not induce early peripheral CD4<sup>+</sup> T-cell responses and lead to persistent infection in chimpanzees experimentally infected with hep-

atitis B virus (HBV). We have now discovered that persistent HBV infection is characterized by the delayed onset of a poorly synchronized, functionally defective intrahepatic CD8<sup>+</sup> T-cell response that is highly activated and that can be surprisingly strong but ineffective. These results suggest that early priming by subviral antigens in high-dose inocula prepares the T-cell response for rapid expansion and functional maturation when the virus appears in the liver. In contrast, when priming occurs intrahepatically after the virus has spread to most of the hepatocytes after a low-dose infection, a poorly synchronized and functionally impaired CD8<sup>+</sup> T-cell response is triggered and a prolonged or persistent infection ensues. These results suggest a hitherto unappreciated role for the superabundant noninfectious subviral antigens present in serum in the outcome of HBV infection.

To test the hypothesis that an early CD4<sup>+</sup> T-cell response is required for clearance of HBV, we infected control and CD4<sup>+</sup> T cell-depleted chimpanzees with the same HBV inoculum and monitored the course of infection. As expected, in the control animals, an acute self-limited HBV infection associated with early peripheral CD4<sup>+</sup> T-cell responses occurred and was followed by a highly synchronized, IFN- $\gamma$ -producing peripheral CD8<sup>+</sup> T-cell response that efficiently cleared the infection. In contrast, in chimpanzees depleted of CD4<sup>+</sup> T cells, no CD4<sup>+</sup> T-cell response to HBV occurred, and a persistent infection developed that was associated with weak CD8<sup>+</sup> T-cell responses with no production of IFN- $\gamma$  similar to the responses induced by the low-dose infections described earlier. Importantly, depletion of CD4<sup>+</sup> T cells at the peak of infection 6 weeks after inoculation had no effect on the kinetics of viral clearance, whereas depletion of CD8<sup>+</sup> T cells at that time prolonged the infection until CD8<sup>+</sup> T-cell numbers returned to baseline. These results indicate that an early CD4<sup>+</sup> T-cell response is required to induce an effective CD8<sup>+</sup> T-cell response to terminate HBV infection.

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## Innate Control of Hepatitis C Virus Infection: Antiviral and Evasion Mechanisms

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G. Cheng, F.V. Chisari

**M**ost of the details of the hepatitis C virus (HCV) life cycle and the host-virus interactions that determine the outcome of infection are poorly

understood, largely because of the lack of tractable cell culture and animal models of HCV infection. Thanks to the recent development of a cell culture model of HCV infection, analysis of all aspects of the HCV life cycle is now possible.

Using that model, we recently found that despite strongly inducing type 1 interferon or interferon-stimulated genes in infected liver, HCV does not induce interferon or interferon-stimulated genes in infected cells. We also found that the viral NS3/4A protease blocks double-stranded RNA signaling by cleaving a key intermediate in the double-stranded RNA signaling pathway. In addition, we discovered a novel NS3/4A-independent mechanism whereby HCV evades the innate host response in infected cells. We also found that interferon rapidly modulates the expression of numerous cellular microRNAs, several of which have sequence-predicted targets within the HCV genome. Synthetic microRNA mimics corresponding to some of these interferon-regulated microRNAs reproduced the antiviral effects of interferon on HCV infection, whereas neutralization of these microRNAs had the opposite effect. These findings indicate a previously unsuspected effector arm of the interferon response that appears to contribute to the control of HCV infection.

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## Hepatitis C Virus Infection and Very Low-Density Lipoprotein

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P. Gastaminza, F.V. Chisari

**I**ntracellular infectious particles of hepatitis C virus (HCV) and precursors of very low-density lipoprotein (VLDL) have a higher buoyant density than their secreted counterparts outside the cell. These biophysical differences suggest that both VLDL and HCV particles acquire lipids while leaving the cell. VLDL synthesis involves the acquisition of cholesteryl esters and neutral lipids by intracellular apolipoprotein B in a process catalyzed by the microsomal transfer protein.

In recent studies, we showed that an inhibitor of microsomal transfer protein and apolipoprotein B-specific short hairpin RNAs prevent assembly of infectious HCV particles and the subsequent maturation and secretion of low-density HCV particles in infected cells. We also showed that as in VLDL biogenesis, presecretory degradation of intracellular infectious HCV precursors via a nonproteasomal mechanism regulates secretion of low-



density infectious HCV particles. These findings suggest that assembly and secretion of HCV particles are tightly regulated by the VLDL metabolic machinery and that only mature, low-density HCV particles are secreted, whereas most newly assembled high-density infectious particles are degraded, implying that acquisition of apolipoprotein B and cellular lipids by HCV imparts a selective advantage as HCV adapts to its natural host.

In ongoing research, we are using mass spectrometry to determine if cell-derived proteins, including components of VLDL and apolipoproteins B and E, are structural components of intracellular and/or extracellular infectious HCV particles. We are also testing the hypothesis that apolipoproteins B and E and other virus-associated cellular factors identified by mass spectrometry play a role in viral entry. We are also studying the role of host cell apolipoprotein E in assembly and egress of HCV particles. Overall, these experiments will provide insight into the role of host cell factors in the HCV life cycle by illuminating the cellular mechanisms that regulate entry, assembly, and egress of infectious viral particles.

## Discovery and Development of Small-Molecule Inhibitors of Hepatitis C Virus Infection

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Using a simple screening assay that reproduces the entire life cycle of hepatitis C virus (HCV) in a miniaturized format and enables simultaneous analysis of the antiviral activity and toxicity of hundreds of compounds, we are screening compound libraries for antiviral activity against HCV. We have discovered 2 novel families of small molecules that profoundly inhibit HCV infection in the low-micromolar range. The nature of the molecules and the click chemistry method used for their synthesis allow rapid preparation of multiple derivatives for analyzing structure-activity relationships. In addition, we discovered a family of peptides that form amphipathic nanotubes that selectively and efficiently inhibit early stages of HCV infection. The identification of novel antiviral molecules and the characterization of their mode of action provide chemical tools to unravel currently unknown

aspects of HCV infection and new compounds for possible development into therapeutic drugs.

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## Toll-Like Receptors in Atherosclerosis

L.K. Curtiss, P.S. Tobias, R. Petrovan, T.A. Bell III, M.R. Richards

Atherosclerosis is a chronic and progressive inflammation of the arterial wall. Hyperlipidemia and infectious diseases implicate innate immune mechanisms as contributors to proatherogenic inflammation. The Toll-like receptors (TLRs) link inflammation, infectious disease, and atherosclerosis. A role for



TLR2 in atherosclerosis was identified in mice deficient in the receptor for low-density lipoprotein (LDLr<sup>-/-</sup>). TLR2 responses to unknown endogenous or unknown endemic exogenous agonists are proatherogenic and are mediated by non-bone marrow-derived cells. In contrast, the TLR2 responses to a defined exogenous agonist such as Pam3 CSK4 are mediated by bone marrow-derived cells. We have therefore verified that TLR2-mediated cell activation in response to endogenous and exogenous agents promotes atherosclerosis.

We used bone marrow transplantation and in situ confocal microscopy of en face aortic tissue segments to precisely map areas of disease predilection within the aortic arch and to measure infiltration of macrophages tagged with green fluorescent protein into the lesser curvature of the ascending aortic arch, a well-defined area of early development of atherosclerotic lesions within the aortic vascular tree. A time-dependent accumulation of bone marrow-derived cells positive for green fluorescent protein was quantified. TLR2 expression was restricted to endothelial cells in regions of disturbed blood flow, such as the lesser curvature region, in atherosclerosis-prone LDLr<sup>-/-</sup> mice. Diet-induced hyperlipidemia increased this regional endothelial TLR2 expression. Accumulation of leukocytes positive for green fluorescent protein, lipid accumulation, generation of foam cells, and injury of endothelial cells in the area of the lesser curvature were all increased by hyperlipidemia, whereas hyperlipidemic double-mutant LDLr<sup>-/-</sup> TLR2<sup>-/-</sup> mice had reduced leukocyte accumulation, lipid accumulation, generation of foam cells, and injury of endothelial cells. This finding is the first one of in vivo site-specific expression of endothelial cell TLR2 and indicates that expression of this receptor on endothelial cells contributes to early atherosclerotic processes in mice in areas of the aorta prone to atherosclerotic lesions.

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## Vascular Thrombosis Transcriptome and Proteome

T.S. Edgington, R. Lin, M. Roychowdhury-Saha

A variety of challenges from infection, immune responses, heredity, injury, and lifestyle conclude with morbidity and mortality. The result most often involves the transcriptome and proteome of the thrombogenic pathway. We are constructing the complete proteome by identifying new alternative-spliced DNA transcripts. Alternative splicing results in a proteome with not only protein isomers but also more divergent proteins. From only 14 genes of group 1 of the thrombogenic pathway, 18 known proteins are encoded. However, 64 other transcripts that encode different proteins have been predicted. Whereas some genes have conservative transcripts, others expand the transcriptome and proteome. We have identified a new class of RNA that appears to regulate splicing of pre-mRNA. This RNA associates with the spliceosome on the chromosomal gene location.

Antisense nucleotides have been identified that rapidly hydrolyze this regulatory RNA modifying the transcriptome. Among the genes affected is the gene for tissue factor, the initiating molecule of the thrombogenic cascade. These alternatively spliced regulatory RNAs enhance the expression of alternatively spliced genes for tissue factor, including the generation of a form lacking the transmembrane domain, resulting in a soluble tissue factor. With a new construct of the transcriptome and proteome, we may reanalyze the molecular pathobiology of the thrombotic diseases.

## New Plays in the Inflammatory Pathways

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Coagulation pathways participate in immune, infectious, and inflammatory responses such as delayed hypersensitivity, immunologic lesions,

and bacterial sepsis, a situation that has propelled exploration of intracellular signaling pathways. The interaction of the complex that initiates coagulation, tissue factor plus factor VIIa (TF-FVIIa), with protease-activated receptors in lethality in models of sepsis implicated the critical role of intracellular signaling. We have found a complex twist in the pathways: a subset of cell-surface annexin 2 molecules acts as a receptor for factor Xa that induces cellular signals involved in the molecular pathobiology of sepsis. The effects of annexin 2 occur in the absence of tissue factor.

Generation of factor Xa by the TF-FVIIa complex is the major coagulation pathway; however, factor Xa can also be generated by the intrinsic coagulation pathway. Perhaps either pathway can evoke the pathogenic cellular responses. We propose that annexin-2 mediates signaling by factor Xa in the absence of cell-surface tissue factor and thus produces the pathophysiologic lethality response. We are investigating the subset of annexin 2 molecules that bind factor Xa and the significance of lipid-raft stabilization by annexin 2 in signal transduction. To study regulation of cell signaling by factor Xa, we are using endothelial cells from mice that lack the gene for annexin 2. Initial *in vivo* studies in a lipopolysaccharide-induced endotoxemia model in mice that lack the gene for protease-activated receptor 1 established the requirement for that receptor. Now, we have found that mice that lack the gene for annexin 2 have decreased thrombin generation. Effects of annexin 2 on survival and inflammatory cytokine generation in this model of sepsis are under investigation.

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## Novel Aspects of the *In Vivo* Molecular Display of Neoangiogenic Tumor Microvasculature

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Using *in vivo* analysis of molecular display of tumor microvascular endothelium, we have identified 340 selectively expressed molecules. These molecules presumably reflect the effects of hypoxia, lactic acid, nutritional deprivation, and neoplastic and host cells. We found that the chemically synthesized 24 amino acid heparin-binding domain (HBDt) of vascular endothelial growth factor selectively binds neuropilin 1 in the complex composed of the receptor for vascular endothelial growth factor and neuropilin 1 on the endothelium in tumors. Selectivity is due to the addition of chondroitin sulfate C. The chondroitin sulfate C may be on serine at position 612 in the b1-b2 bridge and MAM domains of neuropilin 1, but only on tumor vascular endothelium.

The novel hybrid protein of HBDt conjugated to the extracellular domain of tissue factor selectively localized to tumor microvasculature *in vivo*. It was functionally competent to initiate localized thrombogenesis, the intrinsic function of tissue factor, with thrombotic occlusion of tumor vasculature and infarctive eradication of tumor without peripheral thrombosis. *In situ* microscopy indicated participation of platelets. The role of endogenous expression of tissue factor was negated by using genetically modified mice in which expression of tissue factor is only 1%.

## Regulation of Initial Transcript Splicing by $\Sigma$ RNAs

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Initial pre-mRNA DNA transcripts require splicing to excise intronic sequences to generate functional mRNA. Alternative codes exist that result in alternative transcripts. Alternatively spliced transcripts may

result in functional mRNAs that are translated into alternative protein isoforms or even different proteins. We have identified certain large noncoding RNAs as possible regulators of alternative splicing. These RNAs are orthologs of the *alpha* gene in humans, which encodes a nuclear-enriched, large noncoding RNA associated with the splicing factor SC35 domain. This newly designated  $\Sigma$  RNA is highly conserved in vertebrates, lacks DNA repeat elements, is novel, and may have functional significance. The murine ortholog has tissue-dependent expression; expression is highest in brain, thymus, and kidney and lowest in liver, spleen, and testis.

Antisense oligonucleotides inhibited levels of this novel  $\Sigma$  RNA in cell culture. Exon array analyses indicated that RNA posttranscriptional modification, namely alternative splicing, was the major effect, because many of the analyzed transcripts were affected by the antisense oligonucleotide transfection. Validation by Northern blotting, reverse transcriptase–polymerase chain reaction, and DNA sequencing confirmed that alternative splicing of endoglin, QSCN6L, and F3 (tissue factor) mRNAs are influenced by the antisense oligonucleotides. Our results indicate that this first example of a new class of master regulatory RNAs is involved in the composition of the cellular transcriptome via regulation of alternative splicing of pre-RNAs and thereby the cellular proteomes mediating biology and pathobiology.

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## Molecular Biology of Retroviruses

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**W**e study the molecular biology of retroviruses. Our goals are to develop ways to interfere with viral replication and prevent or treat retroviral infections. We use feline immunodeficiency virus (FIV) as a natural model system. FIV causes an AIDS-like syndrome in domestic cats and has structural and functional similarities to HIV, the cause of AIDS in humans. By developing ways to interfere with FIV infection, we hope to devise novel treatments for infections in both cats and humans. In the past year, we continued studies of the molecular characterization

of receptor interactions, the molecular basis for the development of drug resistance, and the role of accessory proteins in surviving the environment of the host. Each study has yielded insights into ways to intervene with the virus life cycle.

### RECEPTOR INTERACTIONS

Like certain strains of HIV type 1, FIV uses the chemokine receptor CXCR4 to infect the target cells, CD4<sup>+</sup> T lymphocytes. However, both HIV and FIV have other primary binding receptors that bind the virus as a prelude to interaction with CXCR4. These primary receptors increase the effective local concentration of the incoming virus by increasing the binding affinity to CXCR4. Interestingly, HIV uses the cell-surface protein CD4 as a primary binding receptor, whereas FIV uses CD134, a glycoprotein expressed on activated CD4<sup>+</sup> T cells. The presence of CD134 on activated T cells explains why FIV can infect and kill CD4<sup>+</sup> T cells, even though the virus does not bind CD4. Thus, an evolutionary conservation of the mechanism of infection by FIV and HIV exists, even though these 2 lentiviruses use different binding receptors.

This 2-receptor mechanism allows the virus to shelter critical regions of the surface subunit of the viral envelope glycoprotein from the immune system until binding and infection have already been initiated. In the past year, we mapped the binding region for CXCR4 on the FIV surface subunit to a 9 amino acid region of the variable loop 3 and identified 3 critical amino acid residues. In contrast, binding of CD134 does not map to a single contiguous epitope; rather it depends on the conformation of the surface subunit for binding.

### DEVELOPMENT OF DRUG RESISTANCE

The aspartic proteases of FIV and HIV are responsible for processing the viral Gag and Pol polyproteins that encode structural and enzymatic proteins of the virus, respectively. The various proteins must be processed from the polyproteins at the proper junctions and also must be processed in the proper order for the virus to be infectious. Thus, the protease is a critical enzyme for the virus and an effective drug target. However, the retroviruses evolve quite rapidly, leading to the development of resistance to drugs and necessitating a parallel evolution of antiprotease drugs to combat drug-resistant viruses, both in FIV and HIV infections.

We have identified critical residues involved in the development of resistance, primarily through the use of chimeric proteases in which amino acid residues in FIV protease have been replaced by HIV amino acid

residues, thus imparting drug susceptibility-resistance patterns of HIV to the FIV background. We are now preparing infectious FIVs that encode chimeric proteases with HIV-like drug specificity to study the development of resistance and protease plasticity. We will continue to use this approach in collaboration with chemists at Scripps Research to develop inhibitors that are less likely to induce resistance.

#### ACCESSORY PROTEINS

Lentiviruses are unique among the retroviruses in encoding accessory proteins that alter the host cell environment to favor viral replication. In FIV, a small protein termed OrfA is required for productive infection of CD4<sup>+</sup> T cells. Without OrfA, the virus can infect the cell, but no virus is produced and the cell does not undergo apoptosis. Although OrfA is clearly beneficial to the virus, the precise function of the protein has been elusive.

To determine the function, we expressed OrfA in feline T cells in culture and then performed microarray analyses on the RNA as a function of OrfA expression. The results indicated that 2 important gene families are downregulated in association with OrfA expression. Certain components of the proteasome/ubiquitin pathway that control degradation of foreign proteins in the cell are downregulated, as are elements of the machinery that controls the splicing of RNA in the nucleus. Components of both of these gene families have been identified as targets for downregulation during HIV infection, albeit by distinct evolutionary mechanisms.

We envision that altering expression of components of the ubiquitin pathway offer the virus a degree of protection from degradation during replication and/or may aid in the co-opting of the endomembrane system to facilitate egress from the host cell. Control of splicing may increase the proportion of full-length viral RNA needed for generation of infectious particles. Future work will involve further dissection of this interesting phenomenon and may yield additional approaches for viral intervention.

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## Epigenetic and Genetic Control of V(D)J Recombination and Formation of the Antibody Repertoire in Normal and Autoimmune Mice

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#### EPIGENETIC CONTROL OF V(D)J RECOMBINATION

In each precursor B lymphocyte, V, D, and J genes recombine to form exons for the light and heavy chains of the antibody molecule. This rearrangement is precisely regulated, and the structure of chromatin (the histone proteins associated with DNA) likely plays an important role in the control of accessibility of V, D, and J genes of the various loci.

Genes in loci undergoing V(D)J recombination are associated with histones that are acetylated. However, many other posttranslational modifications of histones occur. Therefore, we analyzed the extent of methylation of several lysines on histone 3. Methylations of lysines at positions 4, 36, and 79 in histone 3 are associated with active genes, whereas methylation of lysine at position 27 is associated with repressed genes. We found that trimethylation of lysine at position 4 is primarily associated with actively rearranging J genes. In contrast, methylations of lysines at positions 27 (H3K27me3) and 36 (H3K36me2) are the predominant histone modification that occurs at high levels on V genes. The distal and proximal halves of the 2.5-Mb immunoglobulin V<sub>H</sub> locus are under distinct control. Distal V<sub>H</sub> genes do not undergo rearrangement in the absence of the transcription factors Pax5 or YY1 or the Polycomb protein Ezh2.

Ezh2 was of particular interest because it is the methyltransferase that methylates the lysine at position 27 in histone 3. We showed that H3K27me3 was exclusively present on proximal V<sub>H</sub> genes, whereas H3K36me2 had the reciprocal pattern. Furthermore, H3K27me2 was absent in Pax5-deficient mice, thus linking those 2 phenotypes. IL-7 signaling increased the active modification H3K36me2. Pro-B cells in fetal mice preferentially rearrange proximal V<sub>H</sub> genes, and fetal pro-B cells lacked H3K27me3. On the basis of the phenotype of the Ezh2-deficient mice and our data, we propose that the repressive modification H3K27me3,

present on proximal  $V_H$  genes, is necessary to facilitate rearrangement of the distal  $V_H$  genes.

The light-chain loci undergo rearrangement at the pre-B cell stage of differentiation, after heavy-chain rearrangement is complete. Light-chain rearrangement occurs first at the kappa locus and then at the lambda locus. We have shown that this ordered rearrangement is epigenetically controlled. Histone modifications occur first on the kappa genes; they are not present at high levels on lambda genes until the immature B-cell stage, the stage at which receptor editing is initiated. We further found that both the intronic kappa enhancer and signaling through the pre-B cell receptor are necessary to initiate epigenetic modifications at the kappa locus.

#### INFLUENCE OF THE B-CELL REPERTOIRE ON THE FATE OF B CELLS

B cells in the spleen are divided into functionally distinct subsets. We are investigating differences in the antibody repertoires between B cells in the marginal zone, which respond to blood-borne pathogens, and B cells in the follicle, the largest population of splenic B cells. We previously showed that B cells made in fetal or neonatal life lack an enzyme, terminal deoxynucleotidyl transferase, that greatly diversifies the antibody repertoire in adults. We now have evidence that B cells generated early in ontogeny are preferentially selected into the marginal zone of the spleen, suggesting that the fetal or neonatal repertoire of antibodies, which differs greatly from that generated in adults, may be particularly useful against blood-borne pathogens. Furthermore, our recent data obtained by using bone marrow chimeric mice show that preferential selection of B cells with the fetal or neonatal repertoire occurs not only in the marginal zone but also in an earlier transitional compartment. Conversely, selection of the adult-type repertoire occurs preferentially in the follicular compartment, especially in the mature recirculating compartment of B cells.

Our data suggest that the fate of B cells is influenced by the repertoire of the B cells at several branch points during B-cell differentiation. The data also suggest novel alternative pathways of B-cell differentiation.

#### MISREGULATION OF RECEPTOR EDITING IN LUPUS-PRONE MICE

After precursor B cells successfully recombine both heavy- and light-chain gene segments, the cells express a receptor. If the receptor is autoreactive, then the immature B cell continues to undergo light-chain rearrangement until an innocuous receptor is made. This receptor edit-

ing is an important checkpoint in B-cell tolerance. Using B-cell receptor transgenic mice, we found that this process does not function as efficiently in lupus-prone mice as in nonautoimmune mice, and we are investigating the reason for this difference. Such misregulation of this key checkpoint could lead to the release of autoreactive B cells into the periphery, where they can become activated to secrete autoantibodies and cause autoimmune disease.

We are extending these studies to lupus-prone mice in which the B-cell receptor transgenes (i.e., the immunoglobulin light- and heavy-chain transgenes) have been targeted to the endogenous loci. This strategy allows the full extent of receptor editing to occur, and these studies are revealing more aspects of misregulation of receptor editing and of B-cell development in lupus-prone mice.

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## HIV and Microbicides

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In the absence of an effective vaccine, the development of microbicides to prevent sexual transmission of HIV is an urgent need. Topical anti-HIV microbicides are defined as vaginally applied products that prevent HIV male-to-female or female-to-male transmission. The current panresistance to all classes of HIV inhibitors, such as reverse transcriptase and protease inhibitors, is an important concern in the development of topical microbicides that target these enzymes. Thus, identification of novel proteins critical for HIV infection is imperative for the development of microbicides.

Mucosal dendritic cells are the first cells that HIV encounters and may thus play a crucial role in HIV transmission. Interactions between HIV and dendritic

cells are poorly understood. We found that syndecan-3 is specifically expressed on dendritic cells. More importantly, syndecan-3 enhances HIV capture and transmission to T cells by dendritic cells. Thus, HIV exploits syndecan-3 to mediate HIV transmission, and an effective microbicide should target syndecan-3 on dendritic cells to prevent transmission.

We found that the peptide C5A is a potent inhibitor of HIV. C5A corresponds to the N terminus of the membrane-associated nonstructural protein 5A of hepatitis C virus, an essential component of the viral replication complex. Importantly, C5A is virocidal for both hepatitis C virus and HIV. C5A has a broad range of antiviral activity against HIV isolates, and it prevents infection of the 3 *in vivo* cellular targets of HIV by disrupting the integrity of the viral membrane and capsid core. C5A can interrupt an ongoing HIV infection in T cells, and it can prevent transmigration of HIV through genital epithelial cells, infection of mucosal target cells, and transfer from dendritic cells to T cells *ex vivo*, justifying future experiments to determine whether C5A can serve as a novel anti-HIV microbicide.

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## Control of Replication of HIV and Hepatitis C Virus by Cyclophilin

P.A. Galloway, U. Chatterji, J. Mahr, M. Bobardt, S. Selvarajah

Cyclophilins were originally discovered as targets for cyclosporine A. Because cyclophilins have a peptidyl-prolyl *cis-trans* isomerase activity, they have been proposed to act as chaperones in protein trafficking or as catalysts of protein folding. It is well documented that *in vitro* cyclosporine A and derivatives inhibit HIV infection. The current model for the anti-HIV action of cyclosporine A is that it inhibits the binding

of cyclophilin A to the incoming viral capsid protein, an essential interaction for the early steps of HIV replication.

In a recent clinical study, we found that a cyclosporine A derivative only weakly decreased the amount of HIV in the blood, but, surprisingly, it profoundly decreased the amount of hepatitis C virus (HCV). Supporting these *in vivo* data, *in vitro* studies indicated that cyclosporine A and derivatives prevent replication of both RNA and protein in HCV. Recent results suggest that cyclophilins are important for the HCV life cycle, suggesting that they may be the targets for the anti-HCV effect of cyclosporine A. Although one study suggests that cyclophilin B, but not cyclophilin A, is critical for HCV replication, another suggests the exact opposite. Our most recent findings suggest that both cyclophilin A and cyclophilin B are required for HCV replication.

In 2 studies, HCV mutants resistant to cyclosporine A *in vitro* had mutations in HCV nonstructural proteins 5A and 5B (NS5A, NS5B). As for cyclophilin A and cyclophilin B, a similar controversy exists for NS5A and NS5B. Specifically, the results of one of the studies suggest that NS5B has a larger effect on cyclosporine A susceptibility than does NS5A, whereas the results of the other study suggest the opposite. The current model for the anti-HCV action of cyclosporine A and derivatives is that they prevent the binding of cyclophilin A and/or cyclophilin B to NS5A and/or NS5B, an interaction essential for a functional HCV replication complex. An understanding of the mechanisms that control the cyclosporine A inhibitory effect on HCV is imperative because it will result in new anti-HCV treatments and shed light on the still poorly understood early and late steps of the HCV life cycle.

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## Molecular Interactions in T-Cell Development and Activation

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### IMAGING OF MOLECULAR INTERACTIONS IN T-CELL ACTIVATION WITH FLUORESCENCE RESONANCE ENERGY TRANSFER

Interactions between proteins in living cells can be imaged by using Förster or fluorescence resonance energy transfer (FRET), which occurs at distances less than 10 nm. Thus, FRET between fluorescent proteins, for example, between cyan and yellow fluorescent proteins, attached to proteins of interest can be used to investigate interactions between proteins in living cells.

We found that the coreceptor CD8 and the T-cell receptor (TCR) signal-transducing protein CD3 $\zeta$  are recruited to the “immunologic synapse” when antigenic MHC-peptide complexes are presented to the T cell. Using FRET, we showed previously that the TCR and the coreceptor interact solely in the synapse and only when agonist ligands are used. No FRET occurs when weaker ligands (e.g., TCR antagonists) are used. We have compared the TCR-CD8 interaction in cells stimulated with ligands that are at the cutoff between causing either positive or negative thymocyte selection. Negative selectors induce the TCR-coreceptor interaction faster than do positive selectors. A ligand that can act as either a positive or a negative selector has features of the FRET kinetics of both.

We examined the FRET kinetics between CD8 and TCRs containing a mutation in the  $\alpha$ -chain-connecting peptide motif. This motif is a highly conserved sequence close to the transmembrane region of the TCR  $\alpha$ -chain. TCRs with this mutation are poor responders to low-affinity ligands and are defective in positive thymic selection. TCRs lacking the motif are also defective in their ability to bring the TCR into close association with CD8 when stimulated with low-affinity ligands.

We are directly testing the potentially different roles of CD8 $\alpha\alpha$  and CD8 $\alpha\beta$  in formation of the immunologic synapse and the dynamics of association of the kinase Lck with CD8 before and during antigenic stimulation. We are using bimolecular fluorescence complementa-

tion; CD8 $\alpha\alpha$  forms a cyan fluorescent protein and CD8 $\alpha\beta$  forms a yellow fluorescent protein. Both molecules are recruited to the immunologic synapse during antigen recognition. We are trying to identify conditions in which one or the other isotype is preferentially recruited. Interaction with cells bearing the nonclassical MHC class I molecule TL primarily recruits CD8 $\alpha\alpha$ , as would be predicted on the basis of the high affinity of CD8 $\alpha\alpha$ , but not of CD8 $\alpha\beta$ , for TL.

### INFLUENCE OF ENDOGENOUS PEPTIDES IN T-CELL ACTIVATION

We previously discovered that endogenous MHC-peptide complexes help recognition of antigenic MHC-peptides. This finding suggested how T cells can find a few molecules of antigenic MHC-peptide in an ocean of nonstimulatory MHC-peptides. We are now testing whether the CD8 interaction with the MHC molecule is sufficient to provide the aid for antigenic recognition or whether the TCR itself must interact with the endogenous MHC-peptide by using single-chain MHC class I molecules. Preliminary data indicate that the interaction between CD8 and the noncognate MHC class I molecules is indeed sufficient to aid in recognizing limiting quantities of antigen.

### A NOVEL PROTEIN IMPORTANT IN T-CELL DIFFERENTIATION

We have identified a novel protein, thymocyte selection pathway-associated protein, that is expressed primarily in immature, pre-positive selection thymocytes. Mice that lack the gene for this protein have defects in positive selection and in the ability to be stimulated through TCRs. The protein interacts with the proximal T-cell signalosome proteins phospholipase C $\gamma$  and Itk. Thus, the thymocyte selection pathway-associated protein has an important role in T-cell signaling and development. We are determining the function of the protein in the T-cell activation cascade.

### PROTEIN KINASE C $\eta$ IN THYMOCYTE DEVELOPMENT

We previously showed that the  $\eta$  isoform of protein kinase C (PKC) is upregulated during positive selection of developing thymocytes. Of the PKC isoforms, only PKC $\theta$  had been shown to have a special role in T cells, where it is recruited to the immunologic synapse during antigen recognition. The finding that mice deficient in PKC $\theta$  have normal thymic selection suggested that PKC $\eta$  could be replacing PKC $\theta$  in the developing thymocytes.

We found that PKC $\eta$  is also naturally recruited to the synapse in mature thymocytes and T cells. In the absence

of PKC $\theta$ , PKC $\eta$  is expressed at an earlier stage of thymocyte development, where it functions in place of PKC $\theta$ . We produced a strain of mice that lack the gene for PKC $\eta$  and found that the mice have defects in T-cell activation. As in mice that lack the gene for PKC $\theta$ , thymocyte development is normal. In mice that lack genes for both PKC $\theta$  and PKC $\eta$ , positive selection is incomplete.

#### TCR ENDOCYTOSIS, RECYCLING, AND UBIQUITINATION

Because allelic exclusion of the TCR  $\alpha$ -chain is maintained after translation, many mature T cells express 2  $\alpha$ -chain proteins, but few express 2  $\alpha$ -chains on the cell surface. Functional allelic exclusion is attained in the thymus through TCR signaling involving the kinase Lck and the ubiquitin ligase Cbl, which controls degradation of endocytosed TCRs. We are developing a transgenic minigene system to analyze the effects of expression of 2  $\alpha$ -chains on the cell surface. We are also using FRET between ubiquitin monomers and TCR subunits labeled with fluorescent proteins to analyze ubiquitination of TCR after endocytosis.

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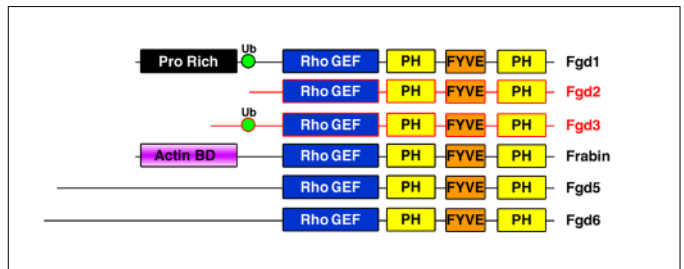
## Functional Roles of Novel Rho Guanine Nucleotide Exchange Factors in Leukocytes

A.L. Gavin, M. Martinic, K. Doyle

**R**ho guanine nucleotide exchange factors (GEFs) are important activators of Rho GTPases. In mammals, the Ras homology (Rho) subfamily of monomeric GTPases, including Rac1–Rac3, RhoA–RhoG, and Cdc42, control such disparate biological activities as growth and cell division, apoptosis, motility, vesicle trafficking, and differentiation. Rho GTPases toggle between GDP-bound inactive and GTP-bound active states, which are regulated by 3 classes of proteins: GDP dissociation inhibitors, GTPase-activating proteins, and GEFs. Rho GEFs catalyze the release of bound GDP, resulting in the formation of the GTP-

bound active protein, able to interact with downstream effector proteins.

The genes for the GEFs Fgd2 and Fgd3 belong to a family identified by relation to the originally discovered gene for Fgd1, which is responsible for a faciogenital dysplasia called Aarskog-Scott syndrome. Although all proteins coded by members of the gene family consist of conserved functional motifs, significant differences at the N termini may play unique functional roles for the protein encoded by each member (Fig. 1). Like many other Rho



**Fig. 1.** Protein domains of Fgd family members. Ub, ubiquitination site; Pro Rich, proline rich; Actin BD, actin-binding domain.

GEFs, members of the Fgd family contain a Rho GEF domain, the so-called dbl homology domain, a pleckstrin homology (PH) domain, a FYVE domain, and a second PH domain. Proteins containing PH domains often bind to phosphatidylinositol-(3,4,5)-trisphosphate, leading to phosphatidylinositol-3'-kinase-dependent membrane recruitment and possible protein conformational changes. In contrast, the FYVE domain often targets proteins to vesicles via interaction with phosphatidylinositol-3-phosphate, which is enriched in endosomes.

We have shown that Fgd2 is associated with endosomes but Fgd3 is not, indicating that although both Fgd2 and Fgd3 are expressed in B cells, they might be active in different cellular compartments. We have also shown that both Fgd2 and Fgd3 can activate Jun N-terminal kinase when overexpressed with Cdc42, but not with Rac1, another Rho GTPase.

Because the genes for Fgd2 and Fgd3 are the only Fgd genes specifically expressed in cells of the immune system, our goal is to investigate the role Fgd2 and Fgd3 play in B-cell signaling and function. We have produced animals deficient in Fgd2 and are producing animals deficient in Fgd3. Of interest, in early studies, we found that compared with their littermates, Fgd2-deficient animals have a weight loss. Thus, in addition to immune cell functions, perhaps Fgd2 plays a role in weight gain and metabolic processes.



## Specificity and Function of Intraepithelial $\gamma\delta$ T Cells

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**W**e have a long-term interest in interactions between intraepithelial  $\gamma\delta$  T cells and their neighboring epithelial cells. We focus on interactions in the thymus, skin, and intestine. We are investigating the development, specificity, and function of these  $\gamma\delta$  T cells. Our results have defined unique properties of these cells and support a specialized role for intraepithelial  $\gamma\delta$  T cells in immune surveillance, wound repair, inflammation, and protection from malignant tumors.

### IDENTIFICATION OF MOLECULES REQUIRED FOR $\gamma\delta$ T-CELL ACTIVATION

In murine skin,  $\gamma\delta$  T cells express an invariant  $\gamma\delta$  T-cell receptor that recognizes an unknown antigen expressed by damaged or malignant neighboring keratinocytes. We have produced soluble skin  $\gamma\delta$  T-cell receptor molecules to detect expression and facilitate isolation and characterization of this unidentified antigen. Using this reagent, we have identified antigen-bearing keratinocytes in wound sites. Future structural studies will determine how these T-cell receptors interact with antigen.

We propose that in addition to antigen, damaged keratinocytes express molecules that participate in activation of skin  $\gamma\delta$  T cells by binding to coreceptors or costimulatory molecules on the T-cell surface. We recently identified several molecules expressed by the skin  $\gamma\delta$  T cells and keratinocytes that provide important costimulatory signals for activation of  $\gamma\delta$  T cells. We found that the semaphorin Sema4D (CD100) is expressed by skin and intestinal  $\gamma\delta$  T cells upon activation. CD100 binds to the semaphorin receptor, plexin-B2, expressed on epithelial cells. We found that interactions between CD100 and plexin-B2 deliver key signals to both the  $\gamma\delta$  T cells and the epithelial cells in the skin and intestine that regulate cell morphology and affect wound healing.

Recent results indicated that the adhesion molecule JAML is uniquely costimulatory for epithelial  $\gamma\delta$  T cells. We identified the coxsackievirus-adenovirus receptor as a ligand for JAML that is expressed on epithelial cells in the skin and intestine. Structural studies of these molecules, performed by our collaborators I.A. Wilson

and P. Verdino, Department of Molecular Biology, indicate a novel signaling model for  $\gamma\delta$  T-cell activation. The results indicate that interactions between JAML and the coxsackievirus-adenovirus receptor play important roles in  $\gamma\delta$  T-cell responses during wound repair and other epithelial challenges.

### ROLES FOR INTRAEPITHELIAL $\gamma\delta$ T CELLS IN EPITHELIAL HOMEOSTASIS AND TISSUE REPAIR

We recently showed a role for skin  $\gamma\delta$  T cells in the reepithelialization stage of wound repair. The  $\gamma\delta$  T cells are activated at wound sites and produce cytokines, including keratinocyte growth factors 1 and 2. In the absence of skin  $\gamma\delta$  T cells, keratinocyte proliferation and tissue reepithelialization after wounding are defective. We also found that skin  $\gamma\delta$  T cells are necessary for the recruitment of inflammatory cells into the wound site. In a novel mechanism, keratinocyte growth factors produced by  $\gamma\delta$  T cells stimulate production of hyaluronan by epidermal cells, which then controls migration of macrophages into wounds.

In previous studies, we showed that intestinal intraepithelial  $\gamma\delta$  T cells play a similar role in responding to tissue damage in a model of colitis. Recent results indicate that in the absence of CD100-mediated signals, increased damage and delayed repair occur because of a lack of production of keratinocyte growth factors, illustrating an important role for signaling through these molecules in  $\gamma\delta$  T-cell functions in the gut. We have characterized a population of invariant  $\gamma\delta$  T cells in nasal mucosa, and studies are in progress to determine roles of intranasal  $\gamma\delta$  T cells in a model of allergic rhinitis. Results in these models support our hypothesis that intraepithelial  $\gamma\delta$  T cells respond to epithelial damage or disease and play important roles in tissue repair and epithelial homeostasis.

Chronic nonhealing wounds are a serious clinical problem because of the increasing numbers of elderly patients and patients with diabetes who have defects in wound healing. We investigated the ability of human T cells isolated from normal epidermis, acute wounds in skin, and chronic wounds to secrete growth factors required for healing. Interestingly, both  $\alpha\beta$  and  $\gamma\delta$  T cells were activated in acute wounds and secreted insulin-like growth factor 1 to promote wound healing. In contrast, in chronic wounds, neither T-cell subset produced the growth factor or could respond to in vitro stimulation, supporting a crucial role for human skin T cells in tissue repair. Further characterization of the functional activity of resident T lymphocytes in acute wounds

should allow the development of new therapeutic strategies against chronic wounds.

Skin  $\gamma\delta$  T cells play roles not only in repair of damaged tissue but also in the normal maintenance of the epidermis. Insulin-like growth factor 1 is required by keratinocytes in the skin for maintenance and during wound healing. We determined that after activation, skin  $\gamma\delta$  T cells produce this growth factor that affects wound healing and apoptosis in the skin. Together these results indicate a role for skin  $\gamma\delta$  T cells in multiple aspects of wound repair and in homeostasis of the epithelium.

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## Mechanisms of Immune Dysfunction in Metabolic Disease

J.M. Jameson, K. Taylor, S. Torng

The number of patients with type 2 diabetes is expected to reach 300 million by 2030. Compared with persons without diabetes, patients with type 2 diabetes are more susceptible to infection and chronic nonhealing wounds. We and others have previously shown that T cells at barrier surfaces play key roles in defending against pathogens and maintaining epithelial surfaces. We are investigating how diabetes negatively affects the immune system, with a special focus on epithelial T-cell populations.

#### CROSS TALK BETWEEN SKIN $\gamma\delta$ T CELLS AND KERATINOCYTES IN DIABETES

Chronic nonhealing wounds are a serious complication of diabetes and have devastating consequences. In murine models of diabetes, decreased levels of insulin-like growth factor 1 and keratinocyte growth factors in wounds contribute to defective wound repair. When these factors are replenished, the markedly delayed wound repair is reversed. Previously, we showed that skin  $\gamma\delta$  T cells play roles in wound reepithelialization via the production of growth factors. Now we are investigating whether decreased levels of growth factors and impaired proliferation/migration of keratinocytes in nonhealing wounds in mice with diabetes are due to dysregulation of skin  $\gamma\delta$  T cells. We have identified a defect in the activation and function of skin  $\gamma\delta$  T cells in the skin of mice with diabetes. Currently, we are determining which

factors associated with diabetes may contribute to skin  $\gamma\delta$  T-cell dysfunction in this disease. Insulin resistance, hyperglycemia, and glucocorticoid inhibition all negatively affect epithelial T-cell function.

#### ROLE OF MAMMALIAN TARGET OF RAPAMYCIN IN $\gamma\delta$ T-CELL DYSFUNCTION

Mammalian target of rapamycin (mTOR) is a metabolic rheostat used by particular cell types to sense nutrients and cytokines. During nutrient overload, mTOR negatively regulates itself, suggesting that it may play a role in the immunosuppression associated with type 2 diabetes. When mTOR is inhibited, susceptible cell types have defects in cell proliferation, survival, and growth. We found that treatment of mice with rapamycin, the inhibitor of mTOR, resulted in defects in proliferation of skin  $\gamma\delta$  T cells and in production of TNF- $\alpha$ . We are now addressing whether this pathway is the one that is inhibited in epithelial T cells in type 2 diabetes. Once we understand how T-cell function in barrier tissues becomes compromised, it may be possible to design therapies that enhance the ability of these immune cells to protect from infection and heal ulcers and chronic wounds.

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## Molecular Regulation of T-Cell Development and Function

J. Kaye, P. Aliahmad, M. Fung, O. Garijo, S. Williams

Precursor cells in the thymus undergo a complex developmental program before seeding peripheral lymphoid organs as mature T lymphocytes. Part of this developmental program includes differentiation of common precursors into helper, cytolytic, regulatory, and innate cell-like subsets of T cells, a process initiated by the specificity of T cells' antigen receptors. We are interested in the molecular regulation of these cell fate decisions. We have focused on a family of nuclear proteins, the original member of which we have shown plays essential roles in T-cell development and lymph node organogenesis. Another family member has been implicated in breast cancer, further highlighting the significance in understanding the function of these evolutionarily conserved proteins.

#### TOX AND T-CELL DEVELOPMENT

We identified thymocyte selection-associated high mobility group (HMG) box protein (TOX) several years

ago. Members of this protein superfamily share one or more copies of a structurally related DNA-binding domain that can recognize distorted DNA and often modify chromatin by bending DNA. In general, HMG box proteins are thought to function as architectural factors that regulate gene expression by promoting formation of transcriptional complexes or by acting as components of chromatin-remodeling complexes.

TOX belongs to a small subfamily of evolutionarily conserved proteins whose members share almost identical HMG box sequences. We found that the TOX HMG box domain can recognize distorted DNA but, unlike other HMG box proteins, is a poor bender of DNA. The inability to bend DNA is due to the lack of a critical internal wedge residue in the HMG box.

Expression of TOX in the thymus is developmentally regulated. To analyze the function of this nuclear factor, we produced mutant mice that globally or conditionally lack expression of TOX. Mice globally deficient in TOX are grossly normal but have a block in development of the major CD4<sup>+</sup> T-cell lineage. In contrast, functional CD8<sup>+</sup> T cells can develop in these mutant mice, yielding insights into lineage commitment in the thymus. Interestingly, loss of TOX also significantly inhibits development of regulatory T cells and natural killer T cells, a finding important for understanding how these distinct cell lineages are formed from common precursors. We recently identified a candidate gene that is regulated by TOX. We are investigating the expression pattern and function of this gene in the thymus.

#### REGULATION OF LYMPH NODE ORGANOGENESIS

During fetal development, lymph node primordia are colonized by hematopoietic progenitor cells, which have been termed lymphoid tissue inducer (LTi) cells. These cells, along with resident stromal organizer cells, are required for inducing lymph node organogenesis. Currently, few transcriptional regulators have been identified that are necessary for LTi development. Surprisingly, mice deficient in TOX lack lymph nodes. Our data suggest that this phenotype is due to a failure of LTi cells to develop. We are working to identify the specific role of TOX in LTi-cell development and the relationship between TOX and other nuclear factors known to be involved in the process.

#### ROLE OF THE TOX FAMILY IN HELPER T CELL DIFFERENTIATION AND DISEASE

We found that TOX is specifically upregulated in follicular T helper cells, a distinct subset of CD4<sup>+</sup> T cells essential for T cell-dependent humoral immune responses. Using T cells derived from conditionally TOX-

deficient mice, we are taking in vivo approaches to determine whether TOX plays a role in the development or function of this important T-cell subset.

Recent reports have identified *TOX3*, the gene for TOX3 as a breast cancer susceptibility locus. Our preliminary data suggest that expression of TOX and TOX3 is quite variable in breast cancer cell lines. We plan to analyze how modulation of these nuclear proteins affects gene expression and behavior of these cells.

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## Regulation of the Innate Immune Response in Inflammation and Infection

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Innate immune cells are the first line of defense in the fight against invading pathogens. We focus on understanding molecular mechanisms that phagocytes and the pulmonary epithelium use to protect the host from microbial injury and how some responses wind up damaging the host. For example, second messengers such as reactive oxygen species (ROS) or nitric oxide that are produced during infection can have beneficial as well as detrimental effects. The overall outcome depends on precise spatial and temporal regulation of these second messengers by the affected cell populations. The intracellular signaling pathways that control these turn on–turn off mechanisms are an ideal target for intervention in disease.

Almost all processes connected to pathogen uptake, pathogen elimination, and sustained inflammation are governed by small GTPases of the Rho family. Our research centers on the Rho GTPases Rac, Cdc42, and RhoA, which are essential regulators for various leukocyte functions ranging from production of ROS to chemotaxis and phagocytosis. Generation of superoxide is accomplished by a Rac-dependent NADPH oxidase (Nox) upon stimulation with chemotactic factors or phagocytic stimuli.

GTPases of the Rho family are also involved in signaling cascades, which originate from pathogen-activated Toll-like receptors. Toll-like receptors 2, 3, and 4 stimulated by microbial products activate Rac1, Cdc42, and RhoA, which regulate pathways required for activation of gene transcription. Currently, we are studying different aspects of signaling by Toll-like receptors in several primary human cell types, including macrophages and neutrophils, and in genetically altered mouse models. We are also examining the impact of this signaling on innate immune cell functions such as upregulation of proinflammatory cytokines, chemokines, and type I interferon.

Another area of research is the interaction and communication between innate immune cells and the pulmonary epithelium. To this end, we have established an *in vitro* reconstitution system for lung epithelium that we use to investigate signaling mechanisms initiated by pathogens. The differentiated and fully functional lung epithelium also serves as a model for studies of lung barrier function and the influence of bacteria-derived ligands and toxins on transmigration of neutrophils. In addition, we are examining processes that lead to uptake of pathogens or environmental particles and the impact of these pathogens or particles on airway epithelial functions.

Recently, ROS-generating Nox proteins have been detected in epithelial cells, and work is in progress to study the molecular basis for ROS generation by these novel proteins. Nox proteins may serve as compartmentalized signaling modules, thereby activating or inhibiting signaling cascades via superoxide, or as an epithelial host defense mechanism via hydrogen peroxide-generating Nox/Duox isoforms. Because of their tissue-specific distribution and distinct localization patterns, Nox proteins might have highly specialized functions and undergo isoform-dependent regulation. For example, Nox4, an oxidase expressed in kidney tissue and melanomas, is constitutively active in certain conditions and does not require any of the known oxidase components for superoxide generation. Elucidating physiologic stimuli and control mechanisms for these Nox proteins combined with structure-function studies will help define the biological functions of oxidases in health and disease.

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## Molecular Mechanisms Regulating Tumor Progression

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J.-D. Lee, R.-Q. Chen, J.-H. Zhu, Y. Wei, Y.-L. Chen,  
Q.-K. Yang, M.-Y. Lai

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**M**etastatic breast cancer (stage IV) cannot be cured. Instead, treatment is mainly focused on maintaining a woman's quality of life while simultaneously lengthening her life. More effective treatment is urgently needed for breast cancer patients with metastatic tumors. Genetic aberrations such as Her2 overexpression in breast tumors are indicators of a poor prognosis: more malignant and metastatic cancers. Understanding the molecular mechanisms that regulate Her2 malignant activity could markedly enhance the effectiveness of breast cancer treatment. Either preventing the progression of breast tumors to a more advanced and/or metastatic stage or stopping or slowing the growth of advanced and metastatic breast tumors would increase patients' survival time and improve their quality of life.

Her2 is a transmembrane receptor tyrosine kinase of the family of epidermal growth factor receptors. Each receptor consists of an extracellular binding domain, a transmembrane lipophilic segment, and (except for Her3) an intracellular functional tyrosine kinase domain. Ligand binding induces homodimerization or heterodimerization of the ErbB receptor family members, resulting in the autophosphorylation of distinct tyrosine residues located within the C-terminal cytoplasmic domain of the receptors. Although it is distinguished from the other 3 Her family members by lack of its ligand, Her2 can heterodimerize with other family members after the family member binds with ligand. Increased expression or mutation of the receptor can also induce dimerization. Once activated, the signal-transduction cascades of these ErbB receptors promote cellular proliferation and survival.

Her2 is overexpressed in 20%–30% of breast cancers and is associated with poor prognosis. Characterization of the regulatory mechanisms of Her2 would provide a platform for generating novel therapeutic strategies that could complement existing anti-ErbB2 treatments, such as trastuzumab (Herceptin).

Her2 transmits its signals into cells initially by phosphorylation of its cytoplasmic signal domain. To investigate regulatory mechanisms of Her2 activity, we screened a phosphatase short interfering RNA library and found that silencing of nonreceptor phosphatase PTPN13 by short interfering RNA markedly upregulated the magnitude of growth factor–induced phosphorylation of the signaling domain of Her2. Our data suggest that PTPN13 attenuated the phosphorylation of the Her2 signaling domain and suppressed the cancerous properties such as proliferation and invasion.

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## Host-Pathogen Interactions: Mechanisms and Applications

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E. Li, D. Scott

To establish parasitism, obligate intracellular microorganisms such as some bacteria and protozoa reside in distinct intracellular compartments.

Pathogens such as *Coxiella* and *Leishmania* survive within the lysosome-like vacuoles, whereas *Toxoplasma* and *Chlamydia* remain within specialized vacuoles, known as parasitophorous vacuoles, or inclusions in the case of *Chlamydia*, for the duration of their replication cycle. Parasitophorous vacuoles are thought to provide a unique environment where the pathogen can efficiently scavenge host nutrients and subvert host cell function. However, little is known about the formation and modification of parasitophorous vacuoles or chlamydial inclusions.

During the screening of bioactive natural products, we found that a crude extract from *Mycelia sterilia*

caused nonfusigenic growth of chlamydiae. The active compound was identified as myriocin, a fungal metabolite previously isolated from several fungi. Myriocin induced nonfusigenic growth at nanomolar concentrations by blocking fusion of inclusions, resulting in accumulation of noninfectious chlamydial progeny. Myriocin, a known inhibitor of serine palmitoyltransferase, interfered with fusion of inclusions by inhibiting de novo sphingolipid biosynthesis; the inhibitory effect was reversed by exogenous sphingosine derivatives but not by ceramides or a sphingomyelin. Together, these results indicate that lipid remodeling is crucial for chlamydial infections.

Infection with *Chlamydia trachomatis* affects more than 140 million persons worldwide and is the most preventable cause of blindness and of sexually transmitted diseases. Infection with *C. trachomatis*, the most frequently reported sexually transmitted infection in the United States and most industrialized countries, and the subsequent consequences disproportionately affect women, especially young women, by causing pelvic inflammatory disease and infertility. Chlamydial infections increase the risk for viral infections such as those caused by herpes simplex viruses, oncogenic papillomavirus, and HIV. Currently, we are focusing on mechanistic studies of how chlamydiae evade the immune system.

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## Macrophage-Associated Protease Network in Cancer and Atherosclerosis

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C. Liu, Y. Liu, F. Guo, Y. Zhang, P. Jiang, D. Xue

Macrophages show marked heterogeneity in their expression of chemokines, surface markers, and metabolic enzymes, such as proteases, in response to local factors. Two types of activated macrophages, M1 and M2, have been operationally defined to represent the extremes of this continuum. M1 or “classically activated” macrophages are induced by proinflammatory mediators such as lipopolysaccharide and

IFN- $\gamma$ . These macrophages have enhanced production of proinflammatory cytokines (TNF- $\alpha$ , IL-6, IL-12) and generate reactive oxygen species such as nitric oxide via activation of inducible nitric oxide synthase. M2 or "alternatively activated" macrophages can be generated in vitro by exposure to IL-4 and IL-13. M2 macrophages have low expression of proinflammatory cytokines, high levels of anti-inflammatory cytokines IL-10 and IL-1 decoy receptor, and enhanced production of arginase and thus promote tissue repair over inflammation. We found that (1) legumain, an activator of matrix metalloproteinase 2 and cathepsin L, and (2) tissue factor, the initiator of the serine protease coagulation cascade, are highly expressed by alternatively activated macrophages. These findings underline the significant influence of the type, M1 or M2, of infiltrating activated macrophages on the balance of the protease network.

#### **CONTRIBUTION OF TUMOR-ASSOCIATED MACROPHAGES TO THE IMMUNOSUPPRESSIVE TUMOR MICROENVIRONMENT**

Tumor-associated macrophages (TAMs) have properties of M2 macrophages and are key producers of growth factors, such as vascular endothelial growth factor, that induce angiogenesis and support tumor cell survival. We found that legumain, an asparaginyl endopeptidase, is overexpressed on the surface of endothelial cells and TAMs in tumor stroma and on neoplastic cells. Using a doxorubicin-based prodrug specifically activated in the tumor stroma by legumain, we showed effective killing of both TAMs and endothelial cells. Massive tumor cell death followed the death of endothelial cells and TAMs. The subsequent collapse of the tumor microvasculature resulted in complete inhibition of tumor growth without any apparent toxic effects.

The prodrug treatment effectively reduced the numbers of TAMs in tumors and resulted in a significant reduction in angiogenic factors. Importantly, prodrug treatment led to a sharp reduction in the number of myeloid-derived suppressor cells positive for the neutrophil marker Gr-1, indicating that elimination of TAMs by the prodrug treatment is sufficient to overcome the immunosuppressive tumor microenvironment and enhance host immune function.

#### **TYPE OF MACROPHAGE ACTIVATION AND THE STABILITY OF ATHEROSCLEROTIC PLAQUE AND ATHEROTHROMBOSIS**

Rupture of a vulnerable coronary atherosclerotic plaque with subsequent coronary thrombosis is the main cause of heart attacks. The formation of thin-

capped vulnerable fibroatheromata is due to a reactive inflammatory response to lipids derived from low-density lipoprotein. Expression of legumain is closely correlated with the formation of vulnerable plaque in humans. We found that M2 macrophages differentiated into foam cells at an accelerated rate when exposed to oxidized low-density lipoprotein. Complexes consisting of legumain and integrins on the cell surface can activate both matrix metalloproteinases and cathepsins, proteases critical for processing and degrading extracellular matrix. Plaque disruption leads to contact between blood and tissue factor and then activation of the coagulation protease cascade. This scenario highlights the role of alternatively activated macrophages in the formation of rupture-prone prothrombotic vulnerable plaques.

The rounds of inflammation and tissue repair associated with plaque probably involve both M1 and M2 macrophages. In mice lacking the gene for apolipoprotein E, we found that atherosclerotic lesions were far more susceptible to forming occlusive arterial thrombosis than were normal vessel walls when exposed to photochemical injuries. Taken together, these data support the role of M2 macrophages in the development of vulnerable plaque and the suppression of legumain and of tissue factor-mediated coagulation as 2 key steps to prevent plaque rupture and thrombosis.

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**Bhattacharjee, G., Ahamed, J., Pawlinski, R., Liu, C., Mackman, N., Ruf, W., Edgington, T.S.** Factor Xa binding to annexin 2 mediates signal transduction via protease-activated receptor 1. *Circ. Res.* 102:457, 2008.

## Role of Protease-Activated Receptor 1 in Cardiac Remodeling and Hypertrophy

N. Mackman, R. Pawlinski, M. Tencati

**T**he role of protease-activated receptor 1 (PAR-1) in myocardial infarction, cardiac remodeling, and myocardial hypertrophy after ischemia-reperfusion injury is unknown and is a rational target for investigation. PAR-1 is the high-affinity receptor for thrombin, the key effector enzyme in the generation of thrombus, the cause of the hypoxic injury and cell death of the myocardium. PAR-1 is expressed by a variety of cell types in the heart, including cardiomyocytes. Moreover, in vitro studies have established that PAR-1 signaling induces hypertrophy of cardiomyocytes.

We found that a deficiency in PAR-1 reduced dilatation of the left ventricle and reduced impairment of left ventricular function 2 weeks after ischemia-reperfusion injury. Compared with wild-type mice, mice that lacked the gene for PAR-1 had increased activation of extracellular signal-regulated kinase 1/2 after ischemia-reperfusion injury. However, PAR-1 deficiency did not affect infarct size. We also found that cardiomyocyte-specific overexpression of PAR-1 in mice induced eccentric hypertrophy (increased dimension of the left ventricle and normal thickness of the ventricular wall) and dilated cardiomyopathy. Interestingly, deletion of the gene for tissue factor in cardiomyocytes reduced the eccentric hypertrophy in mice overexpressing PAR-1.

These results indicate that PAR-1 contributes to cardiac remodeling and hypertrophy. Inhibition of PAR-1 after myocardial infarction may be a novel therapeutic approach to reduce hypertrophy and heart failure in humans after heart attacks.

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## Regulating Adaptive Immunity

M.G. McHeyzer-Williams, L.J. McHeyzer-Williams, N. Fazilleau, N. Pelletier, T. Perkins, L. Mark, S. Okitsu, E. Urich

**W**e seek to understand the mechanism of protein vaccination in vivo. We use model antigen systems to gain experimental access to the cells of the innate and adaptive immune system to understand the molecular control of cellular differentiation in vivo. After vaccination, antigens are processed and presented by a variety of cell types, including dendritic cells and B cells, during different phases of the developing immune response. Recognition of presented antigens by specific helper T cells regulates all facets of adaptive immunity after protein vaccination. These antigen-specific cellular interactions define a series of major developmental checkpoints that critically affect the fate of cells and the emergence of protective immunity.

#### REGULATION OF CLONAL SELECTION IN HELPER T CELLS

Clonal selection is a fundamental process in the development of adaptive immunity. The development of high-affinity memory B cells is central to long-term protection against many pathogens and is controlled in specialized microenvironments called germinal centers. In contrast, clonal selection for helper T cells occurs in the T-cell zones of secondary lymphoid organs and involves direct contact between antigen-presenting dendritic cells and naive T helper cells expressing the appropriate specificity. In earlier studies, we found that this specific recognition event must be greater than a threshold binding affinity to be successful. Surprisingly, changing the dose of antigen did not affect the T-cell receptor-based selection threshold. We now have evidence for the role of vaccine adjuvants in regulating the clonal selection threshold for T helper cells.

Changing the formulation of the adjuvant altered the selection threshold and caused substantial modification in the extent and composition of antigen-specific helper T cells that developed in response to the same dose of antigen. This model system can be used to design a new generation of vaccine adjuvants based on the capacity to promote high-affinity local helper T cell responses.

#### REGULATION OF B-CELL IMMUNITY

Interestingly, promoting high-affinity helper T cells coincides with the development of a specialized compartment of follicular helper T cells. These follicular helper T cells are considered the major regulators of B-cell immunity and link the effect of the vaccine adjuvant on dendritic cell maturation to the development of effector and memory B cells. Our recent studies revealed a new compartment of memory follicular helper T cells that emerge in response to vaccination and remain within the draining lymph nodes. We also found that memory B cells of the same antigen specificity accumulate in the same regions after vaccination. Interestingly, we have strong evidence for persisting cellular sources of antigen that remain locally and have the capacity to activate antigen-specific naive helper T cells. Taken together, these studies reveal a new level of organization for helper T cell memory and B-cell memory that suggests an important function in the surveillance of local portals for antigen entry.

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## Adaptive and Innate Responses to Alloantigens

D.B. McKay, A. Shigeoka, A. Kambo

**S**urgical and medical advances have provided renewed life to patients with end-stage organ disease by replacing diseased organs with healthy ones. Despite remarkable technological advances in transplantation, major immunologic barriers to long-term graft survival still exist. The host immune system must be continually suppressed to prevent rejection of the allogeneic transplanted organ. The suppression of host immunity requires use of nonspecific immunosuppressive medications that are toxic and require lifelong use. We think that advances in transplantation require clarification of the earliest events that occur when a donor organ is transplanted.

In one aspect of our research, we focus on the initial events that regulate activation of the host immune system, especially the events that regulate activation of cells that present donor antigens. Several studies in animals have suggested that the family of evolutionarily conserved cell-surface Toll-like receptors (TLRs) might respond to ligands released by apoptotic and necrotic tissue and that such ligands might be an important molecular trigger for adaptive responses to ischemic injury. We wish to understand how TLR ligation leads to activation of responses against donor antigens and how targeting these mechanisms might prevent allograft recognition and rejection.

Acute allograft rejection requires maturation and migration of immature (passenger) lymphocytes (dendritic cells) from the transplanted organ to draining lymph nodes of the allograft recipient. How the passenger dendritic cells become activated within the transplanted organ is not known; a role for activation by necrotic debris or infectious agents has been postulated. We hypothesized that TLR-dependent signaling is necessary for the initial activation of immature, tissue-resident dendritic cells. We found that the simultaneous absence of the adapter proteins MyD88 and Trif led to inefficient migration of Langerhans' cells from transplanted skin and significant prolongation of skin graft survival, implicating TLR-dependent signaling as a trigger of allograft rejection. Because all transplanted organs undergo variable duration of ischemia during harvest, we recently turned our attention to events associated with ischemia in donor grafts. To further

evaluate TLR-dependent events associated with graft ischemia, we use a model of kidney ischemia-reperfusion injury. Recently, we found that an absence of one TLR in particular, TLR2, conferred almost complete protection from renal ischemia-reperfusion injury, whereas mice deficient in MyD88 or in both MyD88 and Trif had only partial protection. This finding suggested that alternative TLR2 signaling pathways might exist in the kidney.

Interestingly, we also found that TLR2 protein was localized to the basolateral membranes of proximal tubular cells within the kidneys in both mice and humans, cells with the highest susceptibility to low renal blood flow because of a limited vascular supply and an intrinsic high metabolic rate. TLR2 was not detected, in either mice or humans, on tubular epithelial cells of the distal nephron, supporting the hypothesis that the differential tubular epithelial expression of TLR2 also might play an important role in the response of the kidney to ischemic injury. Currently, we are focusing on the mechanisms that contribute to TLR2-mediated injury in the kidney.

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## HIV-Host Interactions: Evolution and Intervention

D.E. Mosier, R. Nedellec, M. Coetzer, J. Salkowitz-Bokal, O. Hartley,\* R. Offord,\* J. Overbaugh,\*\* S. McLaughlin,\*\* J. Mullins,\*\* M. Lederman,\*\*\* H. Hoshino\*\*\*\*

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**H**IV type 1 (HIV-1), the cause of the AIDS pandemic, entered the human population in the 20th century, and both the virus and the host are evolving, rapidly in the case of the virus and slowly in the case of the human host. HIV-1 is a diverse group of related viruses both in individuals and in the global infected population. The most diverse region of the virus is the envelope gene (*env*) that encodes the virus spike. The spike mediates virus binding to human cells and subsequent entry and is also the target for host immune responses. Primary infection with HIV-1 almost



always involves viral spikes (trimers of the envelope proteins) that bind host cell-surface molecules CD4 and CCR5, but the virus can evolve to use other molecules for infection during the course of chronic infection. We are studying this viral evolution and its impact on the sensitivity of the virus to inhibitors that prevent cell entry and the use of alternative coreceptors. In addition, we are developing CCR5 inhibitors as potential microbicides to prevent sexual transmission of HIV-1.

#### **EVOLUTION OF CORECEPTOR USE IN SUBTYPE B HIV-1 INFECTION**

HIV-1 infection usually involves transmission of a single genetic sequence, which then undergoes rapid diversification via mutation and selection. We have evaluated the functional properties of 30 sequential *env* molecular clones from a patient with evidence of coreceptor switching from solely CCR5 to CCR5 and CXCR4 at 5.67 years after infection with subtype B HIV-1. We generated viruses reconstructed from sequential *env* clones obtained from very early in infection until the 5.67-year time point. We found an abrupt and highly significant decline in the ability of viruses to use CCR5 for entry at the last time point, manifested by a 10- to 100-fold increase in susceptibility to CCR5 inhibitors and a reduced ability to infect cell lines with low CCR5 expression. We also found a remarkable 7% divergence in *env* sequences from the most recent common ancestor at 4.10 years of infection; the V4/V5 region had the greatest divergence.

These observations are consistent with a fitness ceiling for Env-mediated entry via CCR5, with a collapse of fitness preceding mutations permissive for use of CXCR4. The stochastic nature of coreceptor switching would thus be influenced by whether or not deleterious Env mutations that significantly impair use of CCR5 occur. These observations on *env* genes cloned directly from an infected patient are consistent with previous reports from our laboratory on functional changes in the envelope protein that occurred during enforced coreceptor switching in vitro. Although changes in the V3 region of *env* were necessary for expansion of coreceptor use, the many changes in the V4/V5 region were essential to preserve virus fitness in the patient.

#### **EVOLUTION OF CORECEPTOR USE IN OTHER SUBTYPES OF HIV-1**

Circulating strains of HIV-1 are divided into subtypes on the basis of significant differences in the viral genome and unique geographic distribution. Infection with subtype C accounts for the highest proportion of the global

epidemic. Expansion of coreceptor use from CCR5 to CXCR4 is rare in infections caused by subtype C but is common in those caused by subtype D. We have examined reconstructed viruses derived from *env* clones from all circulating subtypes for function with CCR5 and expansion of coreceptor use to "nontraditional" alternative coreceptors, that is, members of the chemokine receptor superfamily other than CXCR4. Expansion of coreceptor use to alternative coreceptors (e.g., CCR3, APJ, CMKLR1, CCR8, GPR1, CCR6) is common in non-subtype B HIV-1 isolates and need not be associated with use of CXCR4. The ability to mediate infection via CD4 and CMKLR1 is found solely in non-subtype B isolates and is not correlated with use of CCR3 or CCR5. These results support the concept that each subtype of HIV-1 has evolved to use both CCR5 and alternative coreceptors in subtly different ways and that these different ways contribute to the probability of gaining use of CXCR4.

#### **DEVELOPMENT OF CCR5-TARGETED MICROBICIDES**

We have collaborated with protein chemists to validate the activity of modified versions of CCL5 (formerly termed RANTES), the natural ligand for CCR5. Recombinant molecules that are potent inhibitors of HIV-1 infection via CCR5 have been detected by screening a phage display library. These molecules include CCR5 agonists (i.e., they signal cell activation like native RANTES) and antagonists (no signaling activity) with equivalent subnanomolar potency against a broad range of HIV-1 isolates from all subtypes. These molecules have blocked transmission of SHIV infection (SHIV is a simian immunodeficiency virus with an HIV-1 *env*) in the macaque vaginal challenge model and thus have passed the most stringent preclinical test of efficacy for development as a microbicide against HIV-1.

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## Analysis of Immune Learning in B Lymphocytes

D. Nemazee, A. Gavin, C. Huber, T. Ota, M. Ota, C. Doyle, J. Vela, B. Duong, P. Skog, L. Lim

**T**he main goal of our research is to understand how lymphocytes distinguish between self and non-self antigens. Because antigen receptors on lymphocytes are assembled from component parts through an essentially random mechanism, many cells have self-reactive receptors. Regulation of such autoreactive specificities may be important to prevent autoimmune disease and to ensure efficient response to foreign microbes.

The development of B lymphocytes is a multistep process punctuated by the somatic generation of antibody heavy and light chain genes through DNA recombination, which is catalyzed by the products of recombinase activator gene 1 (*RAG-1*) and *RAG-2*. Because V(D)J recombination is imperfect and error prone, pre-B and B cells are endowed with sensing mechanisms to detect protein expression of heavy chains and assembled heavy and light chains (i.e., intact surface IgM). A major function of the expression of immunoglobulin in immature B cells is signaling to downregulate recombinase activity and to stimulate developmental progression. Newly formed B-cell receptors are also screened for autoreactivity. These quality control mechanisms rely on signaling by antigen receptors.

Previously, we showed that B cells with autoreactive receptors do not downregulate recombination because of excessive signaling through the antigen receptor, resulting in "receptor editing," a process in which previously expressed genes for antibody light chains are inactivated and replaced by secondary DNA recombination. More recent data indicated that editing can also play an important role in inactivating and replacing receptor genes that are underexpressed at the protein level. In this situation, subnormal expression of unligated surface immunoglobulin does not provide a needed signal.

These results suggest that quality control of newly formed B lymphocytes is surprisingly stringent and that through recombinase regulation, B cells are often able to "repair" unacceptable light-chain genes by replacing the unacceptable genes with new genes. Because of the apparent efficiency of the editing process, we suspect that we have uncovered a major cellular "proof-reading" pathway.

A key question of current interest is how signaling through the antigen receptor regulates editing. A major nuclear end point is the regulation of *RAG* transcription. We are assessing the biochemical signaling pathways by which the signal from antigen receptors regulates *RAG* transcription. Recent studies suggested that the transcription factors NF- $\kappa$ B and rel may be involved in both positive and negative regulation of the *RAG* genes. We have also made progress in understanding the triggering involved in B-cell positive selection, in which innocuous B-cell receptors, via tonic signaling, activate a signaling cascade that involves the activity of phosphatidylinositol-3'-kinase and recruited effectors, including phospholipase C $\gamma$ 2 and Akt. This pathway appears to be inactivated in autoreactive immature B cells, a finding that probably explains why the time frame of editing is limited.

To assess the role of receptor editing in preventing unwanted autoreactivity, we have generated mice with a defect in this editing. These mutant mice lack a functional recombining sequence/ $\kappa$  light-chain-deleting element, which is involved in destructive editing of loci for  $\kappa$  light chains in cells that go on to rearrange either a second allele for  $\kappa$  light chains or genes for  $\lambda$  light chains. These mice appear to produce autoantibodies and to accelerate autoimmune disease when crossbred with mice prone to autoimmunity.

In other studies, we have focused on the cues that mature B cells use to distinguish self from nonself. Fully mature recirculating B cells can be rapidly inactivated and induced to apoptosis when confronted with tissue antigen, whereas the same cells are able to respond to antigens expressed by microbes. We are investigating both the death pathway involved in self-tolerance and the nature of the signals that prevent this pathway in responses to nonself antigens. Recently, we found that the ability of B cells to distinguish self from nonself in this setting is independent of T lymphocytes and instead likely involves a novel pathway of self-recognition. We are also testing the hypothesis that immune tolerance in mature B cells depends on specific costimulation by self-tissue, a mode of signaling akin to missing self-recognition by natural killer cells.

Finally, we have reexplored vaccine adjuvants that promote antibody responses. In recent years, immunologists have assumed that the major mechanism of action of adjuvants is activation of the Toll-like receptor signaling pathways. Using mutant mice deficient in the signaling components MyD88 and Trif of Toll-like

receptors, we found that this signaling is largely dispensable for standard immunizations in mice with commonly used combinations of antigens and adjuvants. This research was a collaboration with A. Gavin and K. Hoebe, Department of Immunology and Microbial Science, and B. Beutler, Department of Genetics. The findings suggest the existence of additional adjuvant signaling pathways. Perhaps such putative pathways could be exploited to provide novel ways to boost the efficacy of vaccines.

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## Molecular and Structural Basis of Adenovirus Association With CD46

G.R. Nemerow, L. Pache, S. Venkataraman,\* V.S. Reddy\*

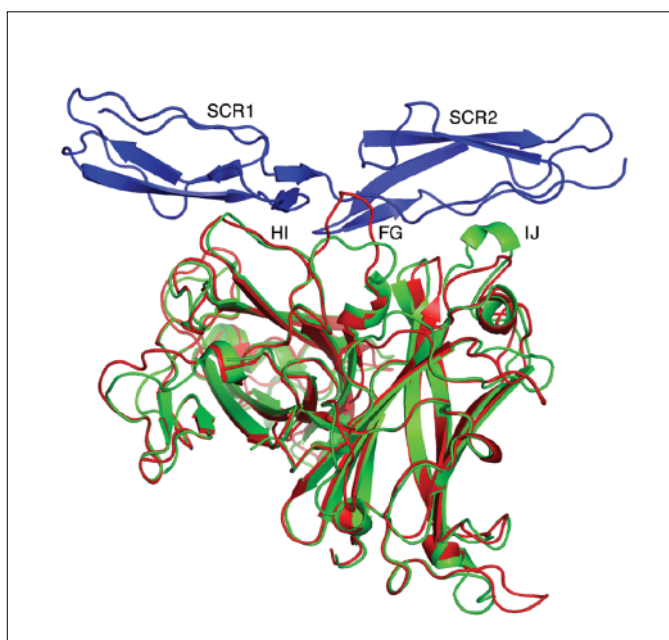
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Adenovirus remains a major vector for gene transfer and vaccine delivery for the treatment or prevention of multiple human diseases. The majority of clinical applications involve the use of adenovirus type 5–based vectors; however, preexisting immunity to this serotype and the ability of type 5 adenoviruses to induce a potent proinflammatory response have hampered widespread use of type 5 vectors. Consequently, alternative adenovirus vectors based on species B viruses, which appear to avoid these problems, are now being explored. Unlike adenovirus type 5, which uses coxsackievirus-adenovirus receptor as its primary receptor, species B1 and B2 adenoviruses (e.g., adenovirus types 11, 35, and 16) use CD46, a member of the family of complement regulatory molecules. Receptor interactions are mediated by the fiber protein, which protrudes from each of the 12 vertices of the viral capsid.

Recently, we compared the molecular interactions of CD46 with 2 B2 species of adenoviruses: 11 and 35. We solved the crystal structure of the adenovirus 35 fiber knob domain at 2.7-Å resolution and constructed a model of the fiber knob in complex with the 2 short

consensus repeat (SCR) domains of CD46 on the basis of a similar model previously reported for a complex consisting of adenovirus 11 and CD46. Although the structure of the adenovirus 35 fiber knob differed subtly (i.e., a shorter IJ loop) from the structure of the adenovirus 11 fiber knob, both fibers bound the receptor in a similar manner and with similar binding efficiencies, as indicated by functional and biochemical analyses. Thus, members of species B2 adenovirus appear to have highly conserved receptor-binding fibers.

In contrast, we discovered that the fiber protein of adenovirus 16, a member of the B1 species, has a markedly reduced affinity for CD46, although virions with the adenovirus 16 fiber can still use CD46 on host cells, albeit with reduced capacity. We determined the crystal structure of the adenovirus 16 fiber knob and found that it has several structural features distinct from those of adenovirus 11 or adenovirus 35 (Fig. 1).



**Fig. 1.** Ribbon diagram of 2 subunits of the adenovirus 16 fiber knob (red) superposed on the complex consisting of adenovirus 11 fiber knob (green) and SCR1 and SCR2 of CD46 (blue). FG, HI, and IJ loops of adenovirus 16 are indicated.

In particular, adenovirus 16 fiber knob has an outer loop, designated FG, that is 2 amino acids longer than the corresponding loops in other B2 adenovirus fibers. Further mutagenesis studies and functional analyses revealed that the longer FG loop of adenovirus 16 likely interferes with CD46 association via steric hindrance. Other types of B1 adenovirus also have this structural feature, a finding that may explain the lower efficiency of CD46 binding by certain adenovirus types. Our recent

studies contribute new knowledge on the molecular basis of CD46 use by various types of species B adenovirus and may point the way to optimizing receptor interactions on target cells with alternative adenovirus vectors.

## Generation of Vault Nanoparticles With Enhanced Cell Delivery Capacity

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**A**lthough use of viral vectors in gene transfer has been successful in some trials, the host immune response and problems with toxicity have limited the use of these agents. In collaboration with L.H. Rome, University of California, Los Angeles, we have inserted the membrane lytic domain of adenovirus protein VI inside naturally occurring cellular nanoparticles known as vaults to increase the ability of the particles to penetrate membranes. We found that the membrane lytic activity of protein VI was retained when the protein was incorporated into vault particles and that these recombinant vesicles could enhance the delivery of a toxin or a cDNA plasmid into murine macrophages in vitro.

These findings set the stage for further exploration of recombinant vault particles as gene/drug delivery devices with reduced toxicity and immunogenicity. The findings should also allow us to more fully explore the membrane-disrupting activity of protein VI.

## Blockade of Adenovirus Cell Entry and Infection by Human $\alpha$ -Defensins

G.R. Nemerow, J.G. Smith

**N**aturally occurring antimicrobial peptides, including the  $\alpha$ -defensins such as human  $\alpha$ -defensin 5 (HD5), make up part of the innate immune response that serves as a first line of protection against invading bacterial pathogens. Defensins are often secreted from mucosal surfaces via specific cell types, such as

the Paneth cells of the small intestine, in response to infection. These peptides can achieve a high local concentration that is sufficient to perturb the lipid envelope of bacterial agents. Interestingly, recent reports indicate that defensins can neutralize a variety of human viruses, including ones that lack an outer envelope, although the mechanism involved in this process has not been characterized.

We found that human defensins HD5 and HNP-1 cause dose-dependent inactivation of adenovirus. These peptides do not block virus binding to cells or prevent internalization of virus. Instead HD5 causes accumulation of adenovirus particles in early and late endosomes, and this accumulation is associated with the failure of virions to undergo partial disassembly and release of the membrane lytic protein VI molecule. This novel mode of virus neutralization may extend to other nonenveloped viruses, including human papillomavirus, that accumulate in endosomes in the presence of HD5. Further studies are under way to examine the molecular and structural features of defensin binding to adenovirus and the immune responses to adenoviruses complexed to defensins.

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## Viral-Immunobiology Laboratory

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**T**he Viral-Immunobiology Laboratory currently encompasses the programs of 3 faculty members: Juan Carlos de la Torre, Dorian McGavern, and

Michael B.A. Oldstone. Each program is independent, but the interactions between the researchers and the use of different technologies provide an intellectual sum greater than any single part. Our studies of both viral and transmissible spongiform encephalopathies (e.g., prion diseases, scrapie) include basic analyses of the mechanisms by which viruses persist, escape immune recognition, and cause disease. Integral parts of the programs are understanding how viruses infect cells; defining the cellular receptors used by viruses; and mapping the trafficking of viruses into and in the cells and the subsequent uncoating, replication, assembly, exit, and spread. Because the immune system has evolved to recognize, attack, and remove these foreign substances, we evaluate the immune response against viruses, probe how viruses subvert this response to provide a selective advantage for their survival, and study how the host can correct this subversion to allow termination of viral persistence.

Other interests include dissecting how viruses and immune cells traffic to the brain and interact there; how viruses are cleared from the brain; and how viruses alter the differentiation processes of cells they persistently infect, thereby disturbing homeostasis and causing disease. We are also investigating how viruses induce autoimmune disease or induce immunosuppression, and we are designing therapies to control viral infection. Because different viruses have different lifestyles, we focus on 4 RNA negative-stranded viruses: Borna disease virus, influenza virus, lymphocytic choriomeningitis virus, and measles virus. We also investigate infectious protein-folding diseases caused by prions.

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## Discovery and Use of Small Molecules That Inhibit Entry of Hemorrhagic Fever Arenaviruses

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**V**iral hemorrhagic fevers caused by the highly pathogenic arenaviruses are among the most devastating emerging human diseases; reported fatality rates in hospitalized patients are 15%–35%. These

viruses include the Old World arenavirus Lassa virus and the New World arenaviruses Junin, Guanarito, and Machupo viruses. Currently, available antiviral therapy is limited, and the only effective vaccine is for infection by Junin virus. A hallmark of fatal arenavirus infections is marked immunosuppression, leading to uncontrolled viremia and death. Those who survive have a vigorous antiviral immune response that controls the infection and leads to viral clearance.

A highly predictive factor for survival is the extent of viremia, which indicates a close competition between viral spread and replication and the host's immune system. Thus, drugs that target viral entry and slow viral spread will provide the immune system a window of opportunity to develop antiviral immune responses and enhance survival. Further, such drugs will be useful for molecular dissection of the pathway of viral spread and replication in infected cells.

We have developed and applied high-throughput screening of libraries of synthetic combinatorial small molecules to identify inhibitors of arenavirus infection. For this screening, we use pseudotyped virion particles bearing the glycoproteins of highly pathogenic arenaviruses over either a Moloney leukemia virus core or a vesicular stomatitis virus core. Through these efforts, we have discovered a series of novel small-molecule inhibitors of viral entry that have closely recognizable core structures and are highly active against pseudotypes of both Old World and New World hemorrhagic arenaviruses. All of these viruses are biosafety level 4 pathogens, the most highly pathogenic for humans, and require the most stringent safety procedures for handling. The antiviral action of the 3 most potent lead compounds were validated *in vivo* against live Lassa, Junin, and Machupo viruses. Interestingly, the identified small molecules were active against both Old World and New World arenaviruses. Because the Old World and New World arenaviruses used have distinctly different receptors (Old World,  $\alpha$ -dystroglycan; New World, transferrin), the viral inhibition was not at the site of binding.

Upon internalization via endocytosis, arenaviruses are delivered to endosomes, where the viruses enter the cytoplasm by pH-induced glycoprotein-mediated fusion of the viral and endosomal membranes. We determined that the antiviral effect of the compounds was inhibition of arenavirus glycoprotein-mediated membrane fusion. Cell-cell fusion mediated by both Lassa virus (Old World) and Junin virus (New World) glycoproteins was affected. An inhibitor concentration

of 200–350 nM resulted in 50% inhibition of membrane fusion, indicating that inhibition of pH-dependent membrane fusion was the major mechanism of the compounds' antiviral activity.

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## Molecular Recognition of the Arenavirus Receptor

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S. Kunz, M.B.A. Oldstone

**T**he receptor for the Old World arenaviruses and clade C New World arenaviruses is  $\alpha$ -dystroglycan, which also serves as a cell-surface receptor for proteins of the extracellular matrix such as laminin. In collaboration with K. Campbell, University of Iowa, Iowa City, we found that specific posttranslational modification of  $\alpha$ -dystroglycan by the glycosyltransferase enzyme LARGE is essential for the functions of  $\alpha$ -dystroglycan as a receptor for extracellular matrix proteins and for arenavirus binding. The N-terminal domain and part of the mucin domain of  $\alpha$ -dystroglycan are critical for the functions of  $\alpha$ -dystroglycan as a high-affinity receptor for laminin and a receptor for arenaviruses.

The binding to  $\alpha$ -dystroglycan by arenavirus is stronger than the binding by laminin or other extracellular matrix proteins; as a result, virus displaces laminin at the binding site. Because  $\alpha$ -dystroglycan is preferentially expressed on dendritic cells compared with other immune cells and dendritic cells contain abundant amounts of LARGE, the basis of arenavirus tropism for dendritic cells is clear. Viral infection of dendritic cells alters the function of the cells, thereby aborting interactions between dendritic cells and T cells and between dendritic cells and B cells necessary to generate an effective and efficient immune response. The result is no or poorly functional antiviral immune cells and persistence of the virus.

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## Therapeutic Vaccination After Reversing the Immunosuppressive Environment Initiated by Viral Persistence

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D.G. Brooks, A.M. Lee, D.B. McGavern, M.B.A. Oldstone

**L**oss of T-cell antiviral function, the hallmark of persistent viral infections, was first detected in an experimental animal model of lymphocytic choriomeningitis virus infection in its natural murine host. Similar, parallel events occur in humans infected with HIV and with hepatitis C or B viruses. Restoration of antiviral T-cell activity is the current goal for treatment of such persistent infections. Unfortunately, vaccines to restore antiviral T-cell activity have routinely been unsuccessful.

Using the lymphocytic choriomeningitis virus model, we found that the lack of success was not due to the vaccine as an immunogen but to the virus-induced immunosuppressive environment. Thus, when we neutralized the major immunosuppressive factor IL-10 by using an antibody to its receptor, we restored the lost T-cell function. When blockage of IL-10 was followed by inoculation with an otherwise previously ineffective therapeutic vaccine, the number of antiviral T cells increased and the antiviral T-cell response was enhanced, with subsequent purging of virus and successful treatment of the persistent viral infection. Thus, we think that current therapeutic vaccination strategies have been unsuccessful in humans with persistent viral infections because the immunosuppressive environment of the host has not been corrected.

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## Facilitation of Opportunistic Infections by Viral Infection: Inhibition of Type I Interferon

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E.I. Zuniga, H. Lewicki, M.B.A. Oldstone

**E**nhanced susceptibility to opportunistic infections during viral infection is a major biomedical and public health problem. We found that lymphocytic choriomeningitis virus infection in its natural murine host diminished the expected ability of plasmacytoid

dendritic cells to secrete high levels of type I interferon upon stimulation of Toll-like receptors. This virus-mediated inhibition of the interferon response was a direct consequence of quantitative and qualitative alterations in plasmacytoid dendritic cells. The result was a decrease in type I interferon and natural killer cell responses. When mice infected with lymphocytic choriomeningitis virus were challenged with murine cytomegalovirus, an opportunistic pathogen, the resultant innate immune defect led to impairment of the host's ability to respond and inhibit the spread of the cytomegalovirus.

Thus, we have initial evidence that viruses can suppress the innate immune response. Such suppression favors the host's enhanced susceptibility to opportunistic infections.

## Defects in Learning and Memory in Scrapie-Infected Mice

M.J. Trifilo, M. Sanchez-Alavez, L. Solfrosi, J. Bernard-Trifilo, S. Kunz, D.B. McGavern, M.B.A. Oldstone

**A**fter infection with RML murine scrapie, transgenic mice expressing the normal cellular prion protein PrP but without its glycosphosphatidylinositol membrane anchor continue to make abundant amounts of the abnormally folded disease-associated protein PrPres but live a normal life span. In contrast, all mice matched to the transgenic mice for age, sex, and genetic background that had a glycosphosphatidylinositol-anchored PrP become moribund and died of a chronic progressive neurodegenerative disease by 160 days after infection with RML murine scrapie.

We found that the infected transgenic mice, although free of the progressive neurodegenerative disease of the cerebellum and extrapyramidal and pyramidal systems, nevertheless had defects in learning and memory, long-term potentiation, and neuronal excitability. Such dysfunction increased over time and was directly associated with an increase in inhibition of  $\gamma$ -aminobutyric acid (GABA) but not loss of excitatory glutamate or *N*-methyl-D-aspartate. Enhanced deposits of abnormally folded infectious PrP protein in the CNS localized with GABA<sub>A</sub> receptors. This localization occurred with minimal evidence of CNS spongiosis or apoptosis of neurons. Monoclonal antibodies coimmunoprecipitated PrPres with GABA<sub>A</sub> receptors. Thus, the clinical defects of learning and memory loss in vivo in the transgenic mice infected with scrapie likely involve the GABAergic pathway.

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## Arenavirus Molecular and Cell Biology: Implications for Novel Antiviral Therapies

J.C. de la Torre, A. Capul, B. Cubitt, S. Emonet

**A**renaviruses are important both as model systems for studies of acute and persistent viral infections and as human pathogens, including several that cause hemorrhagic fevers. Moreover, evidence indicates that the prototypic arenavirus lymphocytic choriomeningitis virus (LCMV) is a neglected human pathogen of clinical importance for which no licensed vaccines are available; current therapy is limited to the use of ribavirin, which is only partially effective and is associated with severe side effects.

Arenaviruses are enveloped viruses with a bisegmented negative-stranded RNA genome. Each genomic RNA segment, S and L, uses an ambisense coding strategy to direct the synthesis of 2 polypeptides. The S RNA encodes the viral glycoprotein precursor and the nucleoprotein; the L RNA encodes the viral RNA-dependent RNA polymerase and the matrix protein Z, a small RING finger protein with key roles in virus assembly and budding.

We have developed a reverse genetics system for LCMV that enables us to re-create all the steps of the virus life cycle from cloned cDNA. This system provides a novel and powerful approach for elucidating the role of viral polypeptides and cis-acting sequences in the control of arenavirus RNA replication, gene expression, and assembly and for identifying and functionally characterizing virus-cell protein interactions that contribute to arenavirus pathogenesis. Using this system, we are developing novel strategies to combat pathogenic arenaviruses by targeting 3 key steps of the arenavirus life cycle: RNA synthesis by the viral polymerase; processing of the viral glycoprotein precursor, which is strictly required for production of infectious progeny; and Z-mediated budding of virus from infected cells.

LCMV is a Rosetta stone in viral immunology and pathogenesis. We can now generate recombinant LCMVs with predetermined specific mutations in their genomes and analyze their phenotypic expression *in vivo*, a novel and powerful approach for elucidating the molecular mechanisms that underlie arenavirus-host interactions, including the mechanisms that facilitate arenavirus persistence and associated disorders.

Thus, we have discovered that the arenavirus nucleoprotein has a strong interferon antagonistic activity. We are determining the mechanisms that underlie this activity and the biological consequences of the activity in virus-host interactions.

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## Virus-Cell Interactions in Persistently Infected Brain

J.C. de la Torre, R. Clemente, B. Cubitt

**P**ersistent viral infections of the CNS can cause progressive neurologic disorders associated with diverse pathologic changes. These findings have led to the hypothesis that viruses can contribute to human mental disorders of unknown etiology.

Borna disease virus (BDV) is an enveloped virus with a nonsegmented negative-stranded RNA genome and is the prototypic member of the virus family Bornaviridae within the order Mononegavirales. BDV is characterized by its noncytopathic multiplication and strong neurotropism and provides an important model for the investigation of immune-mediated pathologic changes and neurodevelopmental and behavioral abnormalities associated with viral infection of the CNS.

The mechanisms that govern the cell tropism and propagation of BDV within the CNS are unknown. We have shown that BDV glycoprotein is responsible for virus receptor recognition and cell entry. Using a genetic screen based on short interfering RNA, we identified 181 cellular genes that may be involved in cell entry of BDV. Proteins encoded by these genes participate in a broad range of cellular functions, suggesting a complex process associated with BDV cell entry. Our initial functional studies have shown that BDV uses a clathrin-mediated cell entry pathway and that successful completion of the BDV entry process requires the integrity of the microtubular network of the cell and participation of endosomal proteases, including cathepsin V. We are also using proteomic approaches to identify cellular receptors that mediate the exquisite tropism of BDV for limbic system neurons.

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# Pathogenesis of Acute and Persistent Viral Infections

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**D**uring the past year, we have focused on 2 important aspects of viral infections: the pathogenesis of acute viral meningitis in real time and novel factors that regulate the pathogenicity of antiviral T cells. Our goal is to clarify our understanding of the interplay between the immune system and host tissues that become virally infected.

## ACUTE INFECTION

To understand viral pathogenesis during an acute infection of the CNS, we recently established an approach in which intravital 2-photon microscopy is used to visualize interactions between virus and the immune system in the meninges. A long list of viruses can induce severe CNS diseases that adversely affect human health. For example, meningitis (or inflammation of the lining of the CNS) is a potentially fatal disease induced by many human pathogens. We postulated that by defining the mediators of the pathogenic process in real time, we would foster the development of novel interventions to lessen or prevent permanent neurologic dysfunction or fatalities.

Intracerebral inoculation of mice with lymphocytic choriomeningitis virus (LCMV) induces severe grand mal seizures and fatal meningitis within 6 days. It is thought that this disease is mediated solely by the activities of cytotoxic T lymphocytes (CTLs). To gain advanced mechanistic insights into the dynamics and function of immune cells in the virally infected CNS, we used intravital 2-photon microscopy to examine disease pathogenesis in real time. We found that fluorescently tagged virus-specific CTLs crossed the blood-brain barrier, entered the subarachnoid space, and engaged virus-infected targets precisely at the time when fatal disease developed. However, deficiency in all known CTL effector mechanisms had no effect on disease, and no disruption of the blood-brain barrier was observed upon CTL extravasation.

Coincident with the arrival of CTLs was a massive influx of myelomonocytic cells (i.e., monocytes and neutrophils). In contrast to the situation with CTLs, extravasation of myelomonocytic cells was associated with profound breakdown of the blood-brain barrier. Neutralization of these cells reduced barrier breakdown and significantly extended survival. Our results suggest that CTLs can mediate a fatal CNS disease by inducing

massive extravasation of innate immune cells across the blood-brain barrier.

## PERSISTENT INFECTION

Because persistent infections affect the immune system in many ways, understanding the network of factors that regulate immune responsiveness is important. In mammalian systems, an extensive immunoregulatory network limits unwanted and potentially fatal immunopathologic changes. However, this regulatory network at times overprotects the host and fosters the development of viral persistence by severely dampening adaptive immunoresponsiveness. Transforming growth factor  $\beta$  (TGF- $\beta$ ) is an immunosuppressive cytokine known to thwart the development of autoimmunity, but its role in the regulation of virus-specific T cells was not entirely understood. To evaluate the role of TGF- $\beta$  in modulating the function of effector and memory antiviral T cells, we virally challenged mice expressing a dominant-negative TGF- $\beta$  receptor type II on T cells. We found that TGF- $\beta$  signaling is important in the development of a virus-specific primary T-cell response. Moreover, when memory T cells with the dominant-negative receptor were therapeutically administered to mice persistently infected with LCMV, the cells caused a fatal immune disorder associated with severe tissue injury. These data collectively indicate that TGF- $\beta$  regulates the development and function of virus-specific T cells at multiple stages and that this cytokine should be considered among the network of molecules that directly regulate the pathogenicity of antiviral T cells.

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## Targeting the Tumor Microenvironment With Minigene Vaccines: A Novel Antitumor Therapy

R.A. Reisfeld, Y. Luo, S. Lewin, T. Cheng, D. Liao, R. Xiang

**A**blation of tumor-associated macrophages (TAMs) remodels the tumor microenvironment and provides a novel strategy for suppressing tumor growth, angiogenesis, and metastasis. We previously established proof for this concept with an oral DNA vaccine encoding the entire gene for murine legumain, an asparaginyl endopeptidase that functions as a highly overexpressed stress protein on the surface of TAMs and tumor tissues. This vaccine, transformed into attenuated *Salmonella typhimurium*, produced antitumor effects by inducing an immune response mediated by CD8<sup>+</sup> cytotoxic T lymphocytes (CTLs) that specifically deleted and killed legumain-positive macrophages, that is, TAMs infiltrating the tumor microenvironment during tumor growth and progression. However, this large, 1.3-kb gene could potentially lead to mutations during vaccine production and also induce immune responses against nonrelevant antigen epitopes that could cause serious side effects. Therefore, we constructed a legumain-based DNA-minigene vaccine that offers a more specific and stable vaccine target.

We constructed mammalian expression vectors encoding either the legumain H-2D or H-2K epitopes with an endoplasmic reticulum signal as a targeting sequence, which directs the protein into the secretory compartment, and a C-terminal peptide for retention in the endoplasmic reticulum. These vaccine peptides were processed in the endoplasmic reticulum and then bound to MHC class I antigen-binding sites to be finally presented to T-cell receptors on the cell surface, inducing a legumain-specific T-cell response. We took advantage of these constructs to create specific minigene vaccines, which did induce an effective T-cell response against breast tumors in syngeneic mouse tumor models.

On the basis of these findings, this same strategy might be applied against different tumors with different genetic backgrounds. This very possibility is of considerable practical importance, particularly because patients have different genetic backgrounds and CTLs display a variety of each individual's genetic background. Inter-

estingly, all our minigene vaccines of different genetic backgrounds induced tumor protection to some extent.

The pLegu-H-2K<sup>d</sup> minigene vaccine was the most effective in protecting mice against D2F2 breast tumor challenges and in preventing pulmonary metastasis. These effects occurred when mice were first immunized with the vaccine and subsequently challenged subcutaneously with murine D2F2 breast carcinoma cells. In contrast, all mice immunized with only the empty vector control had rapid tumor growth. This minigene also induced a strong CTL response capable of killing legumain-positive tumor cells, an effect that was specific, as indicated by the release of IFN- $\gamma$  from activated T cells.

Importantly, cytotoxicity assays clearly showed marked CTL activity only in T cells from successfully immunized mice. Target cells selected to prove this point were those of the murine macrophage cell line RAW, which was previously cultured with IL-4, IL-10, and IL-13 cytokines to induce cell-surface expression of legumain. This step was taken because these cytokines, derived from tumor cells, can switch M1 macrophages to the M2 macrophage phenotype. These findings were verified by Western blot analysis: wild-type RAW cells were legumain-negative; incubation with IL-4, IL-10, and IL-13 rendered them legumain-positive. In contrast, splenocytes from control mice treated solely with empty vector had similar background killing of RAW cells either positive or negative for legumain expression. These data indicate the specificity of the CTL activity induced by the pLegu-H-2K<sup>d</sup> vaccine and the capability of the CTLs to specifically kill legumain-positive TAMs.

We found that tumor antiangiogenesis played a key role in the tumor protection induced by the pLegu-H-2K<sup>d</sup> vaccine. For these studies, we used Matrigel assays in which blood vessel formation was induced within the Matrigel by recombinant basic fibroblast growth factor. When the difference in vessel formation was quantified by measuring the relative concentration of hemoglobin in Matrigel plug extracts obtained from either immunized or control mice, only samples from mice vaccinated with the vaccine had a clear reduction in the mean relative concentration of hemoglobin. This finding was verified by Masson trichrome staining; tissue sections obtained from mice that received the empty vector control had ample and multiple blood vessels. In contrast, the number of blood vessels were markedly reduced in Matrigel sections of samples from mice given

the pLegu-H-2K<sup>d</sup> vaccine. Taken together, these findings indicate that the pLegu-H-2K<sup>d</sup> minigene vaccine induced marked antiangiogenic effects that aided in protecting mice from lethal challenges with D2F2 breast tumor cells.

In summary, the legumain-based minigene vaccines induced effective protection against tumors by ablating TAMs in the tumor microenvironment of our murine breast tumor model. The tumor protection induced by these legumain-K<sup>d</sup> minigene vaccines led to a CTL-mediated attack on TAMs in the D2F2 breast carcinoma tumor microenvironment in syngeneic BALB/c mice. This protective immune response, which specifically killed legumain-positive TAMs, resulted in a marked suppression of tumor growth, metastasis, and angiogenesis. Importantly, the efficacy of the minigene vaccine was relatively similar to that of our previously published vaccine encoding the whole legumain gene, at least in a prophylactic setting. Taken together, these data validate the effectiveness of the first antilegumain minigene vaccine against TAMs in mice and suggest that this strategy could potentially be applied clinically to patients with different genetic backgrounds. Once validated in a therapeutic setting, the minigene vaccine could be a simple, safer, and more flexible alternative to the whole-gene vaccine. This same strategy could add a new dimension to antiangiogenic interventions in cancer immunotherapy and potentially facilitate improved designs and clinical application of DNA-based vaccines in therapy and prevention of breast cancer.

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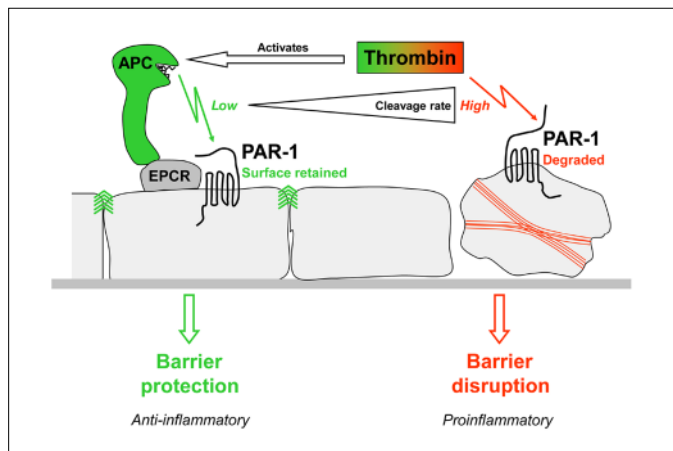
## Protective Signaling by the Protein C Pathway in Endothelial Cells

M. Riewald, R.A. Schuepbach, C. Feistritzer, M. Tencatii

**A**ctivated protein C (APC), an anticoagulant serine protease, is so far the only drug approved for treatment of patients with severe sepsis, but the molecular basis for APC's protective effects is incompletely understood. We have identified a signaling pathway for APC in endothelial cells that requires APC binding to endothelial cell protein C receptor (EPCR) and cleavage of protease-activated receptor 1 (PAR-1). The prototypical thrombin receptor PAR-1 is a member of the G protein-coupled 7-transmembrane domain receptor family. We have hypothesized that PAR-1 evolved to sense the balance of thrombin and APC activity in the endothelial microenvironment.

We analyzed effects of PAR-1-dependent signaling on the integrity of the endothelial barrier because APC has a protective role in conditions, such as sepsis or ischemia-reperfusion injury in the brain, in which the vascular barrier separating the blood from tissues is compromised. PAR-1 signaling by thrombin can have proinflammatory and barrier-disruptive effects, whereas APC-PAR-1 signaling is barrier protective and downregulates the expression of proapoptotic genes. Cross-activation of the sphingosine 1-phosphate receptor 1 is required for endothelial barrier protective effects downstream of APC-PAR-1 signaling. EPCR-bound zymogen protein C is physiologically activated by the thrombin-thrombomodulin complex, and we have shown that this endogenous activation pathway is mechanistically linked to efficient PAR-1-dependent protective signaling by the generated APC.

Thrombin efficiently cleaves and activates PAR-1, and it is difficult to envision how APC-PAR-1 signaling can be relevant in conditions in which thrombin is also present, as in systemic inflammation. To address this question, we have established immunoassays in which we use a panel of monoclonal antibodies to directly analyze the conformation of endogenous endothelial PAR-1 on the cell surface. Recent results indicated that APC-cleaved PAR-1 accumulates on the cell surface even in the presence of thrombin (Fig. 1). In ongoing studies, we are defining the molecular mechanisms that lead to distinct receptor trafficking of thrombin-



**Fig. 1.** Inflammatory disorders such as sepsis are associated with increased permeability of the endothelial cell monolayer at the blood-tissue interface. APC enhances endothelial barrier integrity dependent on binding to EPCR and activation of PAR-1. Thrombin–PAR-1 signaling leads to barrier disruption. However, thrombin also supports barrier protection, because thrombin activates protein C on the endothelial cell surface, and protective signaling by the generated APC is mechanistically linked to activation of protein C. Even though PAR-1 cleavage and activation by thrombin are much more efficient than cleavage and activation by APC, the protein C pathway can mediate barrier protection in the presence of thrombin because APC-cleaved PAR-1 accumulates on the cell surface, whereas thrombin-cleaved PAR-1 is rapidly degraded.

vs APC-cleaved PAR-1. Consistent with the conclusion that PAR-1 can mediate protective APC effects even in the presence of thrombin, APC enhanced barrier integrity upon coincubation with thrombin and reduced vascular permeability in mouse models in wild-type but not PAR-1-deficient animals.

Taken together, our results indicate how balanced regulation can be mediated by a single receptor and support the conclusion that PAR-1 is required for protective signaling by both exogenous and endogenously generated APC. Currently, we are using genetically modified mice in which PAR-1 variants expressed in endothelial cells are efficiently activated by APC but not by thrombin to dissect the *in vivo* roles of thrombin- and APC-dependent PAR-1 activation further. The results will have important implications for future treatment strategies in patients with sepsis and other disorders, including myocardial infarction and stroke, in which vascular barrier integrity and the inflammatory response play key pathogenetic roles.

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## Protease-Activated Receptor Signaling in Cancer, Inflammation, and Sepsis

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### PROTEASE-ACTIVATED RECEPTOR 2 SIGNALING IN REGULATION OF TUMOR ANGIOGENESIS AND CANCER DEVELOPMENT

Proteases are crucial for invasion and metastasis by tumors, but how proteolytic signaling regulates the tumor microenvironment and tumor growth is still incompletely understood. Clinical studies have provided correlative evidence that the expression of tissue factor (TF) and local activation of coagulation in the tumor stroma are predictive of more aggressive cancer phenotypes. However, direct cell signaling by the complex consisting of TF and coagulation factor VIIa through protease-activated receptor 2 (PAR-2) has also been implicated in cancer cell migration and chemokine expression. Because previous studies showed that TF expression enhances tumor growth, we addressed which effector pathways are triggered by tumor cell-expressed TF.

Using prototypic inhibitory antibodies that either block TF-dependent coagulation or direct TF–VIIa–PAR-2 signaling, we found that blocking direct TF signaling is sufficient to attenuate growth of breast cancer. In contrast, inhibition of coagulation had minimal inhibitory effects. TF–PAR-2 signaling induces an array of proangiogenic cytokines, and, consistently, blocking this signaling pathway attenuated local angiogenesis in experimental tumor models. To provide independent experimental validation for the role of PAR signaling in tumor progression, we studied oncogene-driven, spontaneous breast development in mice that lacked the gene for PAR-2. PAR-2-deficient mice, but not mice deficient in the thrombin receptor PAR-1, had a delay in breast cancer progression.

Taken together, these results indicate that tumor progression can be attenuated by selective inhibitors of direct TF signaling. Because traditional anticoagulants, including TF coagulant inhibitors, are associated with a risk for bleeding complications, these findings

suggest a safer alternative to previously tried interventions in cancer patients.

**IDENTIFICATION OF A NODAL POINT THAT CONTROLS SEVERE SYSTEMIC INFLAMMATION AND LETHALITY IN SEPSIS**

Severe systemic inflammatory response syndromes and sepsis remain major causes of mortality in patients of all ages. These diseases are due to an inadequate or exacerbated response of the immune system to pathogens in bacterial infections and viral hemorrhagic fevers. We have begun to unravel the signaling pathways involved in the exacerbated response that leads to death in patients with bacterial sepsis.

We found that proteases of the blood coagulation cascade, thrombin in particular, trigger cellular responses of dendritic cells, key players in both innate and adaptive immunity. Surprisingly, coagulation proteases amplify inflammation not in the vascular system, but rather in the lymphatic system. Thrombin activates PAR-1 on dendritic cells to accelerate their trafficking through lymph nodes. From there, inflammation spreads systemically through the blood stream to cause lung injury and death. Using a combination of genetic mouse models and chemical probes, we found that PAR-1 combines with another G protein coupled-receptor, sphingosine 1-phosphate receptor 3 (S1P3), to amplify systemic inflammation and disseminated intravascular coagulation. Using prototypic drugs that target these G protein-coupled receptors, we provided proof of principle that established sepsis can be interrupted to confine the inflammation locally to the lymph nodes. This intervention is sufficient to prevent systemic inflammation and death due to sepsis.

In ongoing studies, we further characterized how proteases signal to regulate dendritic cell function in immunity, how the dendritic cell PAR-1-S1P3 axis and linked inflammatory pathways can be therapeutically targeted, and how deregulated dendritic cells cause vascular dysfunction. These studies will reveal novel connections between the immune and vascular systems and provide new therapeutic approaches for treating inflammation and cardiovascular complications.

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## Human Intracellular Defenses Against Retroviruses

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With the AIDS crisis well into its second decade, and more 10,000 new HIV infections *each day*, HIV type 1 (HIV-1) has established itself in every human population. With little immediate hope for a vaccine, emphasis has shifted to the use of microbicides and alterations in behavior to at least slow the global progression of this disease. Existing drug regimens have greatly lengthened the life expectancy of AIDS patients. Nevertheless, there is no cure. Difficulties tolerating existing remedies are increasingly prevalent, as is the emergence of drug-resistant strains. The need to identify and understand new vulnerabilities in the HIV-1 life cycle has never been greater.

We have identified a human host cell factor, the *cis-trans* prolyl isomerase Pin1, that can strongly inhibit HIV-1. Pin1 is a ubiquitous host cell protein with well-defined WW protein binding and catalytic *cis-trans* prolyl isomerase domains. HIV-1 replication is boosted by drugs that inhibit Pin1 isomerase activity and by decreases in intracellular Pin1 levels induced by short interfering RNA. These findings suggest that human Pin1 exerts an inhibitory effect on HIV-1 that is apparently alleviated by blocking Pin1 activity. In addition, overexpression of Pin1 in cells that produce infective virions strongly inhibits HIV-1 infectivity at an early step

after cell entry. Point mutants in either the WW or the catalytic domain abolish Pin1 inhibition. We found that Pin1 is a dominant inhibitor in a variety of primate cell lines and that it also inhibits other retroviruses such as simian immunodeficiency virus, HIV type 2, and B-tropic murine leukemia virus. However, a subset of HIV-1 mutants are resistant to Pin1 inhibition.

Our goals are to understand the mechanism of action of Pin1, identify its viral target, determine the step in the viral life cycle it inhibits, and discover how HIV-1 evades total Pin1 inhibition. Because Pin1 is a chaperone that alters the structure of other proteins, we hypothesize that Pin1 is part of a “morphological defense” against retroviruses. Pin1 may alter the shape of incoming antigens to resemble targets that existing restriction factors recognize. This alteration would compensate for the rapid evolution of retroviruses relative to hosts and obviate the need to generate defenses that require anticipating every possible incoming viral antigen. Drugs that boost the activity of Pin1, or attack the same target as Pin1, could provide a new class of antiviral therapeutic agents. Ultimately, understanding the anti-retroviral defenses of host cells will reveal a means of tipping the balance between pathogens and intracellular defenses in the host’s favor.

## Structural Analysis of the Host-Pathogen Interface

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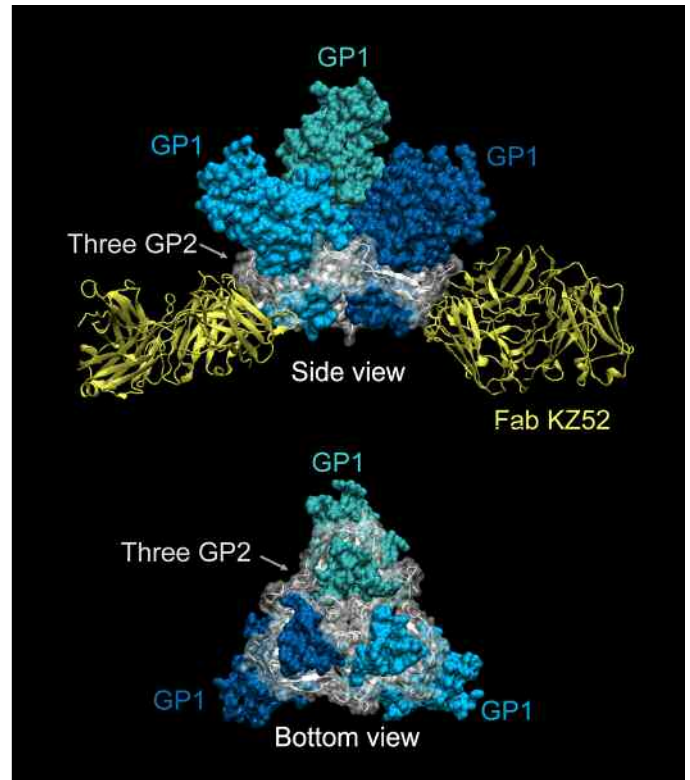
**W**e are crystallizing proteins that play key roles in the pathogenesis and lethality of viral hemorrhagic fevers. The resulting crystal structures will provide (1) information for the design of antivirals and vaccines and (2) structural templates that will enable us to anticipate and rapidly respond to newly emerging and synthetic versions of the viruses and viral proteins.

### EBOLA VIRUS

The genus *Ebolavirus* and the related genus *Marburgvirus* consist of 6 species: *Zaire*, *Sudan*, *Reston*, *Cote d’Ivoire*, and *Uganda ebolaviruses* and *Lake Victoria marburgvirus*. These 6 species cause hemorrhagic fever with 50%–90% mortality. Survival depends on the ability of the host to mount early and strong immune responses. However, such responses are difficult to

achieve. Even those patients who survive often generate little to no neutralizing antibody.

We recently determined the crystal structure of the trimeric, prefusion form of the *Zaire ebolavirus* glycoprotein bound by a neutralizing antibody obtained from a human survivor of hemorrhagic fever caused by this virus. Our 3.4-Å crystal structure reveals that the glycoprotein (GP) resembles a chalice formed by the 3 GP1 subunits, which are responsible for attachment to target cells (Fig. 1). The chalice is encircled and cradled by



**Fig. 1.** Top, side view of the crystal structure of the trimeric, prefusion Ebola virus GP in complex with the human antibody KZ52 (yellow). This structure illustrates that the GP1 attachment subunits (blue) are tethered and surrounded by the GP2 fusion subunits (white). The human antibody, which was derived from a survivor of an Ebola virus outbreak, bridges the 2 subunits together. Bottom, the GP trimeric interface, formed by the intertwined GP2 subunits (white), is visible at the center.

intertwined GP2 subunits, which mediate fusion of viral and host cell membranes in infection. The crystal structure reveals how GP1 forms a clamp on the metastable prefusion GP2 until GP2 is triggered in infection. The human antibody KZ52 binds a prefusion-specific conformation of GP2 at the base of the chalice. Receptor binding sites are recessed in the interior of the chalice bowl, as revealed by cathepsin cleavage in infection. The rest of the GP structure is shielded by a thick glycocalyx, which is likely central to immune evasion and viral sta-



bility. We are now working toward determination of crystal structures of GP from strains of *Sudan ebolavirus* and *Lake Victoria marburgvirus* linked to recent outbreaks of hemorrhagic fever.

Additional structural targets include the viral protein VP35, which blocks innate immune signaling, and the nucleoprotein NP, which is critical for nucleocapsid assembly and transcription. Structural analysis of these proteins will provide insights into viral replication and immunosuppression and will provide the structural basis for the design of antiviral compounds and attenuated strains.

#### LASSA VIRUS

Lassa virus causes an estimated 300,000–500,000 infections per year in West Africa, with a 15%–20% mortality. We are determining crystal structures of the Lassa virus GP spike alone and in complex with the Old World arenavirus receptor  $\alpha$ -dystroglycan. Structural information will explain why concentrations of neutralizing antibody appear to be low in natural infection, will aid in the development of effective treatments, and will provide insight into effective vaccine design strategies.

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## Mechanisms of Antigen Receptor Signaling in Lymphocyte Development and Function

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Lymphocytes defend against infections by recognizing pathogen-derived molecules through antigen receptors (AGRs). Perturbed AGR signaling can lead to immunodeficiencies or to the recognition of innocuous antigens, resulting in allergies or autoimmune diseases. Our core interests are the mechanisms by which AGR signaling directs lymphocyte development and function. By identifying and analyzing novel genes involved in AGR signaling, we hope to improve understanding of the molecular mechanisms that regulate lymphocyte development, function, and malfunction in disease and to contribute to the development of

improved therapies for severe and debilitating immune disorders such as rheumatoid arthritis.

In collaborations with scientists at the Genomics Institute of the Novartis Research Foundation, San Diego, California, we are using various functional genomics approaches, including short interfering RNA screens, haplotype association mapping in inbred mice, and forward genetics to identify the genes involved in AGR signaling and develop hypotheses about their functions. At Scripps Research, we focus on detailed, hypothesis-driven functional analyses of selected genes.

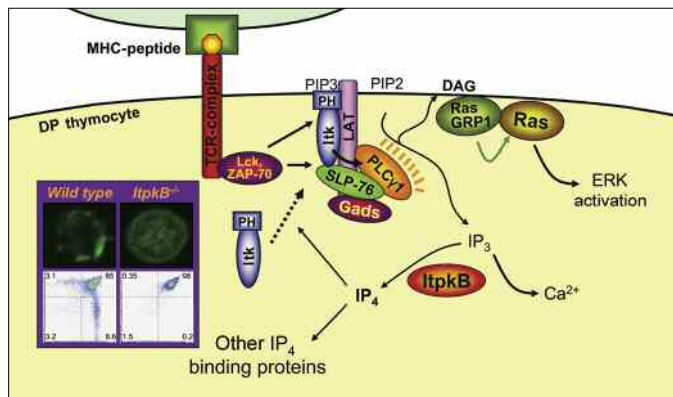
For example, analysis of mutant mice lacking inositol-(1,4,5)-trisphosphate 3-kinase B (ItpkB) led us to the discovery of a novel role for the soluble small molecule inositol-(1,3,4,5)-tetrakisphosphate ( $IP_4$ ) as a positive regulator of PH domains, modules that mediate protein recruitment to cellular membranes by binding to membrane phospholipids. We found that physiologic levels of  $IP_4$  augment binding of several PH domains to their membrane ligand phosphatidylinositol-(3,4,5)-trisphosphate ( $PIP_3$ ). In contrast, T-cell receptor (TCR)-induced membrane recruitment of the protein tyrosine kinase Itk through the PH domain of the kinase is perturbed in *ItpkB*<sup>-/-</sup> double-positive ( $CD4^+CD8^+$ ) thymocytes, which cannot produce  $IP_4$ . These results showed for the first time that  $IP_4$  acts as a “third messenger” in vivo. Because all components exist in all eukaryotes,  $IP_4$  modulation of  $PIP_3$ -binding PH domains likely is a global regulatory mechanism.

Follow-up analyses indicated that this novel  $IP_4$  function is essential for positive selection, a process whereby TCR stimulation drives maturation of functional double-positive cells into T cells. In double-positive cells, *ItpkB* establishes a feedback loop of phospholipase  $\gamma$ 1 (PLC $\gamma$ 1) activation through Itk that is essential for production of the second messenger diacylglycerol (DAG), a critical Ras activator (Fig. 1). This finding established that DAG is essential for positive selection.

Phenotypic differences between mice lacking the gene for Itk and mice lacking the gene for *ItpkB* suggest that  $IP_4$  mediates positive selection through additional targets besides Itk. We are currently identifying these targets to determine how  $IP_4$  controls their functions, in particular, the precise molecular mechanism through which  $IP_4$  regulates the function of Itk PH domains.

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**Fig. 1.** Proposed role for ItpkB in a positive feedback loop sustaining TCR-induced DAG production in double-positive (DP) thymocytes undergoing positive selection. TCR stimulation results in the activation of proximal tyrosine kinases (Lck, ZAP-70); phosphorylation of transmembrane adaptor proteins, including LAT; assembly of signaling complexes centered at LAT; and low-level activation of Itk and its effector PLC $\gamma$ 1. Low PLC $\gamma$ 1 activity allows initial low-level hydrolysis of membrane phosphatidylinositol-(4,5)-bisphosphate (PIP $_2$ ) into the second messengers IP $_3$  and DAG. IP $_3$  mediates calcium mobilization. TCR-induced activation of ItpkB leads to conversion of IP $_3$  into IP $_4$ . IP $_4$  acts as a ligand for the Itk PH domain and augments PIP $_3$  binding, membrane translocation, LAT/PLC $\gamma$ 1 signaling complex interactions, and activation of Itk. These IP $_4$  effects enable Itk to fully activate PLC $\gamma$ 1, allowing DAG accumulation to levels sufficient for membrane recruitment and activation of Ras GRP1. Ras GRP1 in turn mediates activation of Ras and its downstream target extracellular signal-regulated kinase (Erk), essential mediators of thymocyte positive selection. Inset, Perturbed Itk localization to sites of TCR engagement in ItpkB $^{-/-}$  double-positive thymocytes, shown via confocal immunofluorescent microscopy, coincides with a block of T-cell development at the CD4 $^+$ CD8 $^+$  double-positive stage, shown via flow cytometric analysis.

## Consequences of T-Cell Recognition of Self-Antigens and Tumor Antigens in Normal and Diabetes-Prone Mice

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**T**he consequence of antigen recognition by naive CD8 $^+$  T cells can be either tolerance or immunity, depending on the activation status of the antigen-presenting dendritic cells and the duration of exposure to antigen. Understanding the signals that result in either T-cell deletion or immunity is important in preventing autoimmunity, which is a failure to control self-destructive T lymphocytes. This understanding is also important in promoting tumor immunity, in which

the goal is to promote the autoimmune destruction of tumor cells. We are comparing the consequence of the interaction of naive CD8 $^+$  T lymphocytes with a self-antigen expressed by the insulin-producing beta cells in the pancreatic islets in 3 different types of mice: normal mice; nonobese diabetic (NOD) mice, which are diabetes prone; and mice in which the beta cells express an oncogene that promotes spontaneous transformation and production of tumors.

### MECHANISMS OF PROTECTION FROM TYPE 1 DIABETES BY GENETIC POLYMORPHISMS

The spontaneous diabetes that develops in NOD mice is similar to type 1 diabetes in humans. The disease process involves destruction of the insulin-producing beta cells in the pancreas by CD8 $^+$  T lymphocytes. In both humans and mice, genetic regions have been identified in which allelic polymorphism predisposes individuals to type 1 diabetes. We are studying the effects such allelic polymorphism, designated *insulin-dependent diabetes (Idd)* loci, has on the establishment of CD8 $^+$  T-cell tolerance.

Congenic mice that express protective alleles at *Idd3/5* have normal abortive activation of islet antigen-specific CD8 $^+$  T cells in the pancreas, suggesting that tolerance is restored at the earliest time when naive CD8 $^+$  T cells first encounter antigen. In contrast, in NOD mice, such CD8 $^+$  T cells accumulate in the pancreatic lymph nodes and then enter the islets. This difference in the accumulation of CD8 $^+$  T cells in the pancreatic lymph nodes occurs in the absence of all CD4 $^+$  T cells. Production of radiation bone marrow chimeras suggests that *Idd3/5* genes that determine tolerance are expressed by a nonlymphoid bone marrow-derived cell, possibly the antigen cross-presenting dendritic cells.

Another congenic NOD strain expresses protective alleles at the *Idd9* locus. Islet-specific CD8 $^+$  T cells accumulate in the pancreatic lymph nodes of *Idd9* mice, similar to the situation in other NOD mice. The survival of activated self-specific CD8 $^+$  T cells in *Idd9* mice is limited, however, compared with the survival of such cells in NOD mice, indicating that compared with *Idd3/5* genes, *Idd9* genes correct a distinct checkpoint of tolerance.

### ENHANCEMENT OF TUMOR IMMUNOTHERAPY BY SYNERGY BETWEEN ADJUVANTS TARGETING INNATE AND ADAPTIVE IMMUNITY

Self-tolerance is a major barrier to effective immunotherapy because the tolerance results in the elimina-



tion of most self-reactive CD8<sup>+</sup> T cells, including a subset of cells specific for tumor-expressed antigens. The tumor-specific CD8<sup>+</sup> cells that remain escape tolerance because they have low avidity for tumor cells. Strategies that enhance the effector capabilities and longevity of tumor-specific CD8<sup>+</sup> cells would greatly enhance tumor immunotherapy. Cytokine complexes composed of IL-2 and antibodies to IL-2 are a highly effective reagent for augmenting CD8<sup>+</sup> T-cell activity. However, the cells only survive as long as they are in the presence of the cytokine complexes. When the complexes are removed, the cells soon die of cytokine withdrawal. By combining IL-2 complexes with an inflammatory adjuvant, polyinosinic acid–polycytidylic acid, we have been able to both greatly enhance the effector function of low-avidity tumor-specific T cells and promote survival of the cells long enough to achieve tumor eradication. This strategy presents many of the benefits of whole-body irradiation, including the provision of high levels of homeostatic cytokines, enhanced expansion of effector cells relative to regulatory T cells, and provision of inflammatory cytokines, and is therefore likely to be a strategy for both tumor vaccines and adoptive immunotherapy of cancer.

#### ROLE OF CD4<sup>+</sup> HELPER CELLS IN PROMOTING TUMOR CELL DESTRUCTION BY CD8<sup>+</sup> CELLS

CD4<sup>+</sup> helper T cells can enhance the performance of CD8<sup>+</sup> T cells in different ways, including enhanced clonal expansion during activation of CD8<sup>+</sup> T cells, enhanced tissue infiltration by the activated effector CD8<sup>+</sup> cells, and enhanced survival of the effector cells. We are assessing the effects of CD4<sup>+</sup> helper T cells at various times after activation of CD8<sup>+</sup> T cells to evaluate the ability of the helper cells to promote destruction of tumor cells by CD8<sup>+</sup> cells. One way CD4<sup>+</sup> T cells help tumor-specific CD8<sup>+</sup> cells is by facilitating the entry of the CD8<sup>+</sup> cells into the tumor tissue. This process is much less efficient if no CD4<sup>+</sup> helper T cells are present within the tumor environment. In addition, we found that the lytic activity of CD8<sup>+</sup> cells in the tumor is greatly augmented by the presence of CD4<sup>+</sup> helper cells. The cellular and molecular interactions that result in such enhancements are currently under investigation.

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## Regulation of Homeostasis of Mature T Cells

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The overall size and composition of the pool of mature T cells are relatively stable in young adults because of control via homeostatic mechanisms. Recent research has indicated that 2 related cytokines, IL-7 and IL-15, are the central homeostatic signals for mature T cells. Hence, survival of naive T cells, which mostly persist in interphase under normal conditions, is supported by these 2 cytokines together with T-cell receptor signaling from contact with self-peptide–MHC ligands. Memory T cells, which persist at a higher state of activation than naive T cells do and periodically undergo basal homeostatic proliferation, depend solely on the 2 cytokines for persistence. Under normal conditions, the 2 cytokines are thought to be produced constitutively by non-T cells, such as epithelial, stromal, and antigen-presenting cells, and sustain survival of a finite number of T cells. The 2 cytokines are present at very low levels under normal conditions, probably because of continuous consumption by T cells. With severe depletion of T cells, the levels of the 2 cytokines increase because of lack of consumption, and because these cytokines are mitogenic for T cells at high concentrations, the lymphopenic conditions can trigger remaining T cells to enter spontaneous cell division, a condition known as acute homeostatic proliferation.

The acute homeostatic proliferation observed under most lymphopenic conditions drives naive T cells to undergo slow cell division, once every 24–36 hours, and gradually induces the dividing cells to acquire features of memory cells. This finding probably reflects that IL-7 and IL-15 are upregulated only at moderate levels under most lymphopenic conditions and/or that these 2 cytokines are only weakly mitogenic for naive T cells. In stark contrast, we have found 2 conditions in which highly upregulated levels of IL-2 and IL-15 can induce naive

T cells to proliferate at a prodigious rate, resembling cells activated by foreign antigens.

The specific conditions occur in mice deficient in the expression of 1 of the IL-2 receptor chains, specifically the CD122 (IL-2R $\beta$ ) and CD132 (IL-2R $\gamma$ ) chains. Hence, when normal naive CD8<sup>+</sup> T cells are adoptively transferred into hosts deficient in CD122, the donor cells undergo massive proliferation and rapid differentiation into effector cells, closely mirroring the T-cell response to foreign antigens. Similarly, naive T cells undergo massive proliferation upon adoptive transfer into CD132-deficient mice, but the proliferating cells acquire characteristics of central memory cells rather than those of effector cells.

The strong proliferation in the 2 types of hosts does not occur in the absence of MHC molecules, suggesting that the responses are driven by self-ligands. Moreover, the variability in the acquired differentiation state of the expanding T cells, effector vs central memory, appears to be a result of the dominance between IL-2 vs IL-15 in the 2 types of hosts. Hence, the IL-2 level is probably higher than the IL-15 level in CD122-deficient mice, which have lymphadenopathy from expansion of activated T cells that produce IL-2. In contrast, CD132-deficient mice are severely lymphopenic due to a defect in thymopoiesis and probably have higher levels of IL-15 than of IL-2. In both instances, IL-2 and IL-15 likely are upregulated because the absence of either CD122 or CD132 precludes the expression of the receptors for these 2 cytokines.

In summary, naive T cells can be induced to undergo acute homeostatic proliferation of variable speed, even in the absence of lymphopenia. For a few members of the common  $\gamma$ -chain (CD132) family of cytokines, the speed of proliferation depends on the particular cytokine involved and its level of availability.

Despite the increasing understanding of T-cell homeostatic cytokines, clinical use of the cytokines is yet to be realized. One major problem is that exogenous cytokines injected systemically do not have the expected high activity, presumably because of their extremely short in vivo half-life. One approach to overcome this difficulty is to inject the cytokine after it is bound to a monoclonal antibody specific for the cytokine.

We and others have shown that complexes composed of IL-2 and an IL-2-specific monoclonal antibody have 100-fold more biological activity upon injection than does IL-2 alone. Similarly, we found that the biological activity of IL-7 in vivo is greatly

increased by association with a monoclonal antibody to IL-7. Under in vivo conditions, complexes of IL-7 and its antibody had 50- to 100-fold higher activity than did free IL-7 and induced massive proliferation of pre-B cells. The IL-7-antibody complexes also increased thymopoiesis in normal mice and restored thymopoiesis in IL-7-deficient mice. For mature T cells, complexes composed of IL-7 and the IL-7-specific monoclonal antibody induced marked homeostatic proliferation of both naive and memory CD4<sup>+</sup> and CD8<sup>+</sup> cell subsets, even under normal T cell-replete conditions. Finally, the IL-7-antibody complexes were able to enhance the magnitude of the primary response of antigen-specific naive CD8<sup>+</sup> cells. The strong stimulatory activity of the complexes could be useful for treatment of immunodeficiency and cancer.

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## Structure-Function Studies of Innate and Adaptive Immunity

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#### ACTIVATION OF T-CELL RECEPTORS

**O**ur goal is to understand the molecular switches that govern the initiation of T-cell activation. We have achieved assembly of functional complexes of T-cell receptors (TCRs) on artificial bilayers with recombinant forms of TCR $\alpha\beta$ , CD3 $\delta\epsilon$ , CD3 $\gamma\epsilon$ , and CD8 $\alpha\beta$ .

We use single-molecule, multicolor imaging by total internal reflection fluorescence microscopy, in collaboration with K. Fish, University of Pittsburgh, Pittsburgh, Pennsylvania, and D.P. Millar, Department of Molecular Biology, and electron microscopy to examine the dynamics and membrane relationships of each subunit within the complex. To understand the dynamic relationships between the different constituents of the TCR complex, we use MHC ligands displayed in solution or at the surface of polystyrene beads and liposomes.

Interactions of MHC and TCR molecules with their respective membranes and their neighboring molecules could provide simple switches essential for T-cell activation. This hypothesis is supported by our structure determination, in collaboration with A.K. Mitra, University of Auckland, Auckland, New Zealand, of the structure of an MHC molecule attached to a phospholipid bilayer that shows parallel orientation of the long axis of the molecule with the lipid leaflet. In collaboration with I.A. Wilson, Department of Molecular Biology, we are determining 3-dimensional structures of CD3, TCR complexes, and CD8 $\alpha\beta$ .

#### AUTOIMMUNE DIABETES

We are using MHC multimers to detect antigen-specific T-cell populations in nonobese diabetic mice, which are diabetes prone. Pathogenic T cells are characterized by analyzing cytokine secretion and use of TCRs by single cells. We are also trying to treat type 1 diabetes by deleting antigen-specific T cells in vivo during the preclinical phase of the disease. For this therapy, we are using MHC molecules to deliver doxorubicin liposomes to autoreactive cells. The specificity of the intervention will limit side effects and complications of general immunosuppression. We have just completed the first structure determination of a complex consisting of a TCR and a diabetogenic MHC molecule. On the basis of the structure, we have established the first comprehensive hypothesis linking the single  $\beta$ 57 MHC class II polymorphism to immune diabetes. In collaboration with B. Jabri, University of Chicago, Chicago, Illinois, we have extended the same observations to human autoimmunity and HLA-DQ8.

#### LINKS BETWEEN INNATE AND ADAPTIVE IMMUNITY

We are using biophysical methods to examine lipid binding to CD1 to determine the factors that govern the presentation of lipids to T cells. A family of lipid transfer proteins known as saposins, which are involved in the catabolism of lipids, are critical for the loading of natural glycolipids onto CD1 and the selection of

natural killer T cells. Other lipid transfer proteins such as Niemann-Pick C1 and C2 molecules or GM2 activator protein are also involved in the loading of endogenous and exogenous ligands. In collaboration with A. Bendelac, University of Chicago, and P.B. Savage, Brigham Young University, Provo, Utah, we are using RNA interference, genetic techniques, and recombinant biochemistry to study CD1 within the context of lipid metabolism. At a structural level, we are examining recognition of dissimilar ceramides such as  $\alpha$ -galactosylceramide and isoglobotrihexosylceramide ( $\beta$ -linked) by a TCR bearing a unique  $\alpha$ -chain ( $V\alpha$ 14 in mice,  $V\alpha$ 24 in humans) and a limited set of  $V\beta$  partners. We are also investigating the adjuvant properties of natural and synthetic ligands for natural killer T cells to develop new vaccination approaches.

#### INNATE IMMUNE RECEPTORS

Recognition of unique features of the prokaryote world is embedded in a series of receptors of the innate immune system called pattern recognition molecules. Each of these receptors can sense the presence of a family of unique prokaryotic compounds such as glycolipids, proteoglycans, DNA, or RNA and allow activation of macrophages, dendritic cells, and neutrophils. We are collaborating with R. Ulevitch, Department of Immunology and Microbial Science, to decipher the structural basis of this mode of recognition. We expressed recombinant forms of receptor family members from *Drosophila*, mice and humans to compare the biophysical and structural characteristics of the receptors and to delineate new activation pathways. We have also produced monoclonal antibodies against each of these molecules for biological and structural studies.

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## Predisposing and Effector Genes in Systemic Autoimmunity and the Role of T-Cell Homeostasis in Autoimmunity and Cancer

A.N. Theofilopoulos, D.H. Kono, R. Baccala, R. Gonzalez-Quintal, M.K. Haraldsson, D. Wichramarachchi, R.M. Chintalapati, C.A. Louis-Dit-Sully, B.R. Lawson

**W**e have continued our research on topics related to spontaneous pathogenic autoimmunity, as occurs in systemic lupus erythematosus (SLE or lupus), and induced beneficial autoimmunity, as occurs in cancer. In studies on pathogenic autoimmunity, we use both forward and reverse genetic approaches to identify predisposing and effector genes in murine models of lupus, and we address potential means to correct defects in T-cell homeostasis in this disease.

### A LUPUS-SUPPRESSING MUTATION OF THE CORONIN-1A GENE

Susceptibility to lupus is largely dependent on genetic predisposition. Therefore, identification of disease-modifying genes is central to understanding the etiology and pathogenesis of lupus and to further advancing diagnosis, prognosis, and treatments. Because lupus is highly heterogeneous in humans, we and others have used mouse models of various spontaneous and induced forms of the disease to identify pertinent genes. Using genome-wide approaches and polymorphic markers, we identified numerous predisposing loci distributed throughout the autosomal chromosomes in 4 major lupus-prone strains of mice: NZB, NZW, BXSB, and MRL. We then created interval-congenic strains for several loci to define component phenotypes, reduce the interval in which predisposing genes are located, and ultimately clone the specific genes. Interval congenics thus far include the NZB/NZW-related *Lbw2*, *Lbw5*, and *Lbw7* (chromosomes 4, 7, and 1), the MRL/B6-*Fas*<sup>lpr</sup>-related *Lmb1*–*Lmb4* (chromosomes 4, 5, 7, and 10), and the NZB/DBA/2-related *Hmr1* (chromosome 1). Currently, we are producing smaller interval, subcongenic strains to more precisely localize the *Lbw2* locus on chromosome 4.

We also cloned the *Lmb3* locus and discovered that it is a spontaneous nonsense function-impairing mutation of the gene for coronin-1A unique to our C57BL/6-*Fas*<sup>lpr</sup>/Scr mouse colony. Among the 7 mammalian coronins, only coronin-1A is expressed almost

exclusively in hematopoietic cells. The protein inhibits formation of F-actin filaments by sequestering the nucleation-promoting actin-related protein 2/3 complex in its inactive open form and by serving as a cofactor for disassembly of F-actin mediated by cofilin and actin-interacting protein 1. The disease-suppressing allele of the gene for coronin-1A was associated with accumulation of cellular F-actin in lymphoid cells and with impaired migration, increased apoptosis, and reduced antigen receptor-mediated activation of T cells that correlated with a defect in calcium flux between release of stored calcium and activation of the calcium release-activated calcium channel. Suppression of autoimmunity was due to decreases in T-cell number and function, which specifically impaired T cell-dependent, but not T cell-independent, humoral responses. Interestingly, mice deficient in other actin and cytoskeletal regulatory proteins have a similar T-cell defect in calcium flux. These findings provide impetus for investigating the role of actin-regulatory proteins in autoimmunity and their potential as therapeutic targets.

### IDENTIFICATION OF EFFECTOR GENES IN AUTOIMMUNITY

Recently, we began a new project to identify effector genes in spontaneous and mercury-induced systemic autoimmunity. The method we use, random mutagenesis with *N*-ethyl-*N*-nitrosourea followed by phenotypic screening, does not require preselection or even knowledge of the genes beforehand. During the next few years, this approach may result in the identification of a significant proportion of the genes, many of which are likely to be novel and unanticipated, as was the gene for coronin-1A, which was not previously suspected to play a role in lupus.

### SIGNALING BY TOLL-LIKE RECEPTORS IN LUPUS

Innate immune responses are primarily induced through signaling by the evolutionarily conserved Toll-like receptors (TLRs). Recent evidence strongly suggests that microbial pathogens and even endogenous (self) molecules can induce autoimmunity via the engagement of TLRs, particularly TLRs that recognize nucleic acids, such as TLR3 (double-stranded RNA), TLR7 (single-stranded RNA), and TLR9 (hypomethylated DNA). Accessibility to these endosomal TLRs by self-nucleic acids is thought to be mediated by the complexing of these molecules with lupus-associated autoantibodies with corresponding specificities.

We have further defined the role of these TLRs in lupus pathogenesis by creating lupus-prone mice con-

genic for the 3*d* mutation of the gene *Unc93b1*. This mutation was recently identified by our collaborators B. Beutler and associates, Department of Genetics, through germ-line mutagenesis with *N*-ethyl-*N*-nitrosourea. This mutation impairs signaling by all 3 TLRs that recognize nucleic acid, antigen cross-presentation by class I MHC molecules, and, in part, exogenous antigen presentation by class II MHC molecules. The 3*d* mutant C57BL/6-*Fas*<sup>lpr</sup> and BXSB congenic mice had significantly decreased serologic, cellular, and histologic disease characteristics and increases in survival, firmly establishing the role of nucleic acid-recognizing TLRs in the pathogenesis of lupus. Synthetic blockers for these TLRs may be a new class of therapeutic agents for lupus.

#### **TYPE I INTERFERONS AS PATHOGENIC EFFECTORS IN LUPUS**

We and others have obtained evidence that type I interferons promote disease expression in the New Zealand strains of lupus-prone mice. We examined the role of type I interferons in the pathogenesis of lupus in BXSB mice, another lupus-prone strain. Lupus develops early in life in male BXSB mice that have a large X to Y chromosome translocation and *Tlr7* gene duplication. BXSB mice deficient in IFN- $\alpha$  receptor subunit 1 (IFNAR1) had significantly reduced disease, indicating that the pathogenic effects of TLR7 hyperexpression are mediated by the production of type I interferon.

Because deletion of the gene for IFNAR1 affects the development of a diverse set of immunocyte subsets, determining the therapeutic effects of IFNAR blockade when used after the development of lupus and at different stages of the disease is important. This determination can be accomplished by using either recombinant soluble receptors or antibodies. The antibody approach could not be used for long-term treatment because the available antibodies to this receptor were of rat origin and would be ineffective if they elicited an immune response by the host. Recently, our collaborators, R. Schreiber and colleagues, Washington University School of Medicine, St. Louis, Missouri, used DNA plasmids encoding mouse IFNAR1 as the immunogen in mice genetically deleted of this receptor and produced a mouse monoclonal antibody to IFNAR1, a development that allowed us to examine the therapeutic effects of IFNAR blockade. Treatment of male BXSB mice with the antibody at early stages of lupus led to significant reduction in the manifestations of autoimmune disease and extended survival. These findings, together

with encouraging preliminary results in mice treated at later stages of lupus, provide further support for pursuing such treatments in humans who have lupus.

#### **INHIBITION OF LYMPHOPROLIFERATION AND LUPUS BY BLOCKADE OF IL-7 RECEPTORS**

We have posited that lupus is essentially a disease of lymphocyte homeostasis and have further suggested that an excess of T cell-trophic cytokines, such as IL-7 and IL-15, may drive proliferation of autoimmune T cells. To address this hypothesis, we examined T-lymphocyte phenotypic markers, expression of receptors for T cell-trophic cytokines, and the potential therapeutic effects of antibodies to these receptors in MRL-*Fas*<sup>lpr</sup> mice.

We found that phenotypically CD4 and CD8 T cells of these mice resembled T cells associated with homeostatic proliferation rather than T cells associated with nominal antigen-induced proliferation. Furthermore, double-negative cells that lacked the CD4 and CD8 coreceptors and excessively accumulated in Fas-defective mice were almost completely devoid of receptors for IL-7 and IL-15.

We reasoned that the downregulation of cytokine receptors on this major T-cell population might reduce cytokine "sinks," providing increased availability of survival- or proliferation-mediating resources for newly emerging autoreactive T cells. Indeed, adoptive transfer of CD4 or CD8 single-positive T cells into older MRL mice with accumulation of lymphocytes resulted in proliferation of the transferred cells, suggesting an excess of cytokines in these mice. Consequently, we examined the effect of blocking antibodies to IL-7 receptors at either the early or the late stage of lupus. We found that treatment with antibodies to IL-7R reduced lymphoproliferation, dermatitis, kidney disease, and mortality. Thus, blockade of receptors for cytokines that promote T-cell survival and proliferation is likely to be an effective treatment in autoimmune and lymphoproliferative syndromes.

#### **SUPPRESSION OF SYSTEMIC AUTOIMMUNITY BY INHIBITION OF TRANSMETHYLATION**

The functional repertoire of proteins is highly enlarged by posttranslational modifications of the proteins, among which transmethylation is a major process. We previously found that inhibition of transmethylation reduces T-cell activation by interfering with arginine methylation of Vav1, the essential guanine-exchange factor in T-cell receptor signaling, particularly in CD4 T cells. These findings suggested that inhibition of transmethylation might be an effective means to treat

both organ-specific and systemic autoimmune diseases, in which CD4 T cells are indispensable participants. Long-term treatment, however, could not be considered unless potent reversible transmethylation inhibitors were developed.

In collaboration with C. Yuan, Diazyme Laboratories, Poway, California, we showed the efficacy of a recently developed reversible inhibitor of S-adenosyl-L-homocysteine hydrolase in experimental autoimmune encephalomyelitis, the accepted model of multiple sclerosis. More recently, we extended these studies to the MRL-*Fas*<sup>lpr</sup> lupus model and found that treatment initiated at either the early or the late stage of lupus resulted in increased survival and significant reductions in all disease parameters. We are further defining the mechanistic aspects of this treatment.

#### T-CELL HOMEOSTATIC PROLIFERATION AND TOLERANCE TO TUMOR ANTIGENS

The role of excess signaling by T cell-trophic cytokines in spontaneous autoimmunity suggests that the preferential expansion of self-reactive T cells observed with such stimulation could be exploited to induce beneficial autoimmunity to cancer cells. Thus, presentation of tumor-derived antigens in the presence of cytokine excess might be a means to decrease the activation threshold of tumor-specific T cells, induce preferential proliferation of the cells, and promote effective antitumor responses.

Previously, we showed that in melanoma-challenged mice, depletion of lymphocytes provokes homeostatic proliferation of T cells and that this proliferation was associated with an effective antitumor autoimmune response. We therefore examined whether this approach could also be used to treat more aggressive malignant tumors, particularly metastatic breast cancer. Confirming our results with melanoma, we found that lymphopenia-induced homeostatic proliferation of T cells significantly inhibited growth of subcutaneously induced breast carcinomas. Surprisingly, however, lung metastasis and mortality were both exacerbated in the treated mice, suggesting that depletion of lymphocytes may also favor metastatic disease, probably by eliminating immunocytes that constrain metastatic spread during the early phases of lymphocyte recovery. These findings prompted us to explore alternative ways to induce proliferation and activation of T cells and to apply this information to cancer immunotherapy.

On the basis of the observation that the *in vivo* effect of IL-2 is dramatically enhanced if this cytokine

is administered as a complex with specific antibodies, in collaboration with C.D. Surh, Department of Immunology and Microbial Science, we examined whether the same *in vivo* enhancement was applicable to IL-7, a main cytokine in homeostatic proliferation. Whereas IL-7 or IL-2 injected alone had no effects, immune complexes of these T cell-trophic cytokines significantly enhanced proliferation and accumulation of CD4 and CD8 T cells in nonlymphopenic mice. Importantly, in mice challenged with metastatic breast carcinoma cells, IL-7-antibody complexes, and, in particular, IL-2-antibody complexes significantly reduced lung metastasis and mortality. These immune complexes were effective therapeutically against established tumors and were dependent on activation of CD8 T cells. Additional studies with T-cell receptor transgenic cells showed that IL-2-antibody complexes induce activation of cytolytic effector functions and reverse anergy in antigen-specific CD8 T cells. Thus, immune complexes of lymphotropic cytokines effectively induce T-cell proliferation, activation, break of tolerance, and therapeutic antitumor responses in a model of aggressively metastatic breast carcinoma.

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## Toll-Like Receptors and Innate Immunity

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P.S. Tobias

**M**y colleagues and I focus on 2 aspects of Toll-like receptors (TLRs) and innate immunity: involvement of TLRs in atherosclerosis and pharmacologic inhibitors of TLR signaling.

In collaboration with L.K. Curtiss, Department of Immunology and Microbial Science, we are defining the effects of TLR gene deficiencies in atherosclerosis-prone mouse strains. The most detailed data we have are on the role of TLR2 (see report of Dr. Curtiss).

Generally speaking, TLR initiation of inflammatory cascades is involved in pathogen detection, in which

TLRs initiate antipathogen homeostatic responses, and in sterile inflammation, such as atherosclerosis and ischemia-reperfusion injury in the kidney (see report of D. McKay, Department of Immunology and Microbial Science). These conditions could reasonably be called autoimmune diseases of the innate immune system.

To begin to develop pharmacologic agents that might be useful in further characterization of TLR function and possibly as therapeutics, we have developed a high-throughput screening assay to detect the earliest functional interaction between TLRs and their proximal signal-transducing adapters. Using this assay, we have identified a dozen small-molecule, water-soluble, cell-permeable compounds that inhibit responses to TLR2 and TLR4 ligands. We are expanding the scope of this assay to more TLRs and to other innate immune receptors, particularly NOD1 and NOD2.

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## Viral Pathogenesis and Antiviral Immunity

J.L. Whitton, N. An, N. Benning, C. Cornell, S. Crocker, B. Eam, R. Feuer, R. Frausto, P. Krogstad, S. Harkins, C. Kembal, F. Liu, J. Whitmire

#### ANTIVIRAL T CELLS: REGULATION OF QUALITY AND QUANTITY

**C**D8<sup>+</sup> T cells play a key role in combating most viral infections, either by killing virus-infected cells or by showering the cells with antiviral cytokines such as IFN- $\gamma$ . During microbial infection, epitope-specific CD8<sup>+</sup> T-cell responses usually exist as a hierarchy; responses to some epitopes are strong, or dominant, whereas responses to other epitopes are weak, or subdominant. The hierarchy is regulated by a poorly understood phenomenon termed immunodominance.

We have found that immunodominance depends on expression of IFN- $\gamma$ . Our current hypothesis is that the immunodominance hierarchy (i.e., the relative abundances of the various epitope-specific T-cell populations)

is defined by the rate at which the various epitope-specific cells can initiate production of IFN- $\gamma$ ; the fastest cells become the dominant population during the primary immune response. Recent data indicate that expression of receptors for IFN- $\gamma$  on CD8<sup>+</sup>T cells is tightly regulated and that cells lacking these receptors are at a selective disadvantage. In the absence of this receptor, the frequency of memory cells after viral infection is decreased approximately 100-fold. Therefore, evolution appears to have used IFN- $\gamma$  to kill two birds with one stone; the cells that are best suited to combat viral infection (i.e., the cells that most rapidly elaborate IFN- $\gamma$ ) are the cells that are preferentially expanded in the host during primary infection and preferentially enter the memory pool. These studies of T-cell regulation have been extended to include CD4<sup>+</sup> T cells.

In most studies of T-cell function, including ours, synthetic peptides are used to stimulate T-cell responses *in vitro*. We have developed a novel method to identify T cells, and other cell types, that are actively responding to contact with authentic antigen *in vivo*. This method not only will be useful for studies of immune responses to infection but also may facilitate a better understanding of autoimmune disease. For example, we have identified autoreactive cells that actively produce cytokines *in vivo* during experimental allergic encephalitis, a mouse model of multiple sclerosis in humans.

#### VIRAL PATHOGENESIS

Coxsackievirus B3 is an important human pathogen that causes a variety of clinical syndromes, including myocarditis and pancreatitis. We previously showed the importance of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the control of viral infection and in the immunopathologic etiology of virus-induced disease. However, coxsackievirus B3 does not induce strong CD4<sup>+</sup> or CD8<sup>+</sup> T-cell responses, despite reaching very high concentrations in various tissues. To understand the reasons for this finding, we have prepared several recombinant forms of the virus that express well-characterized CD4<sup>+</sup> and CD8<sup>+</sup> epitopes, and we have evaluated the *in vivo* presentation of these epitopes by using epitope-specific T cells as indicators.

We found that coxsackievirus B3 presents CD4<sup>+</sup> T-cell epitopes but does a good job of blocking the presentation of CD8<sup>+</sup> epitopes. Furthermore, the virus appears to accelerate cellular endocytosis, denuding the cell membrane of MHC class I molecules. We postulate that other proteins, such as cytokine receptors, also may be removed from the cell surface. Thus, the virus makes the infected cells invisible to T cells and

untouchable by cytokines, key effectors of the host's antiviral response. We will further investigate these phenomena, and we will determine why CD4<sup>+</sup> T-cell responses are also weak despite the detectable presentation of related epitopes by coxsackievirus B3.

#### **AUTOIMMUNITY**

Together with colleagues at the University of Utah and at La Jolla Institute of Allergy and Immunology, we are studying the molecular basis of autoimmunity induced by viral infection. Some autoimmune diseases (e.g., multiple sclerosis) appear to be triggered and/or exacerbated by a wide variety of viral infections. Two general mechanisms, molecular mimicry and bystander activation, have been proposed to explain this phenomenon. We have suggested an alternative explanation that is based on changes in antigen presentation that occur during almost all viral infections.

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