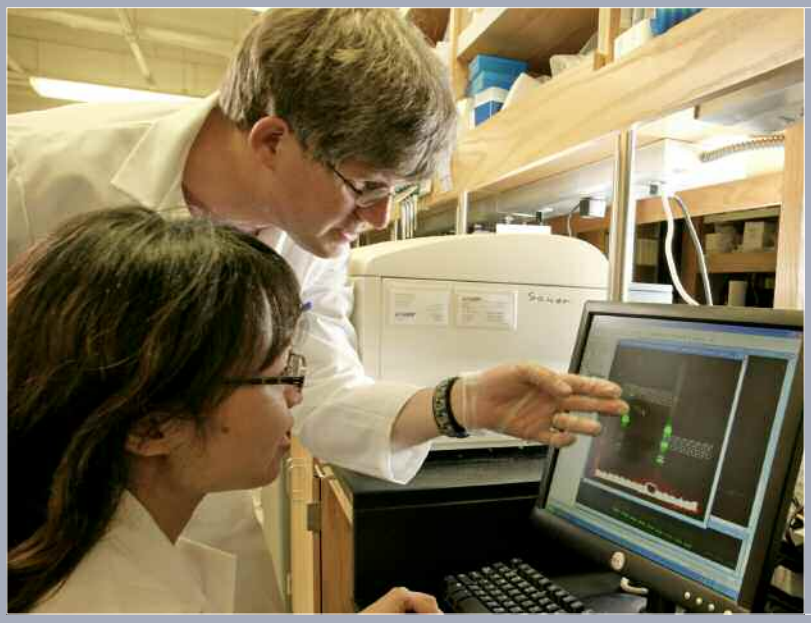
Immunology

Crystals of the Ebola viral glycoprotein in complex with a human antibody derived from a survivor of the 1995 Kikwit, Zaire, outbreak and of the antibody alone. Work done by Jeffrey E. Lee, Ph.D., research associate, in the laboratory of Erica Ollmann Saphire, Ph.D., assistant professor.



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** Appointment completed; new location shown*

*** Joint appointment in the Department of Cell Biology*

**** Joint appointment in the Department of Molecular Biology*

***** Appointment completed*

Richard Ulevitch, Ph.D.

Chairman's Overview

This past year has made the innocuous acronyms A1 and A2 ever more ominous. Without meaning to most outside the world of those who apply for grants from the National Institutes of Health (NIH), these terms indicate frequent scenarios facing scientists who depend on NIH funding. A1 and A2 reflect a seriously broken grant review process and restricted funding. The requirement for highly productive and successful investigators to resubmit applications for continuation R01 grants is almost a certainty. Thus, the former model of scientists at Scripps Research obtaining funding in a predictable manner has been drastically changed.

In the United States, biomedical research has always been a valued part of governmental support and is well recognized as the source of new drugs, vaccines, diagnostics, medical devices, and so on. The net gain to the healthcare system far exceeds the amount of money invested in the discovery of these items. However, each of us is now faced with a period in which the crisis is impeding the progress of biomedical research in this country. This block in progress will have considerable short- and long-term national and international consequences. I have not seen a worse time to apply for funding from NIH.

Here, in one of the best departments of immunology, scientists must spend more and more of their time writing and rewriting grant applications and finding alternative funding sources. And institutional resources are also more and more limited because of excessive demands. One consequence is an exodus from academic science. Another effect is migration of scientists from "soft money" institutions to those offering "hard currency." To my sincere regret, such a migration is occurring in our department at a much higher rate than in the past. And as chairman, I cannot counter offers from other institutions offering funded positions to our faculty.

The NIH responses to concerns from the research community range from calling for input from scientists about how to "fix" the review process (i.e., another committee to eventually write a report) to more grandiose proposals emanating from the top administration of NIH. In 35 plus years at Scripps Research and more than a decade as chairman of the Department of Immunology, I have not observed such a dire situation. I hope to see more encouraging news soon, but I have nothing to report now.

Despite this difficult environment, researchers in the department continue to achieve the highest levels of scientific productivity; outstanding training of predoctoral and postdoctoral fellows; and success in obtaining funding, albeit with increasing delays in funding.

One highlight of the past year was the award to Charles Surh of the prestigious Ho-Am Prize in Medicine. The Ho-Am Foundation, set up in memory of the late Lee Byung-Chull, founder of Samsung Group, presents awards annually to those who have made outstanding contributions to science, culture, and human welfare. Dr. Surh was recognized for his work on the selection of young T cells in the thymus and the factors that control survival of mature T cells in the extrathymic environment. He received the award at a ceremony on June 1, 2007, in Seoul, Korea.

Another important award was received by Bruce Beutler, who retains his close ties to the Department of Immunology while leading the newly formed Department of Genetics at Scripps Research. Dr. Beutler received the 2006 William B. Coley Award for Distinguished Research in Basic Immunology from the Cancer Research Institute. This award recognizes his seminal contributions that have defined key steps in pathways of the innate immune system. He uses forward genetics to identify key genes in host processes essential for com-

bating a variety of pathogens. The award ceremony was held at the Rainbow Room in New York City on June 27, 2006.

During 2007, the department has obtained funds from NIH to purchase a 2-photon microscope. Under the leadership of Nick Gascoigne, we were successful in obtaining about 80% of the needed funding; Scripps Research Administration provided the balance. This facility is now fully operational, and we hope to see its continued use. Training for new users is provided, and we hope to acquire upgrades in a timely fashion. This facility provides our investigators with state-of-the-art technology to advance their research programs.

Another measure of success of the department comes from "The 2005 Faculty Scholarly Productivity Index, by Academic Analytics," published in the Journal of Higher Education. In short, the Department of Immunology was ranked second behind Yale University for our graduate program in immunology. The criteria for ranking include the following as the most important variables: publications, which can include the number of books and journal articles published as well as citations of journal articles; federal-grant dollars awarded; and honors and awards. So, despite the serious deficits in the NIH grant review system and in NIH funding, the department continues to excel.

I hope that in the next year our faculty will continue to provide the best in discovery science, education, and participation in the many aspects of science that exist outside the laboratory. In return, Scripps Research will continue to provide maximal levels of support, needed and deserved. As always, it is a great honor and pleasure to serve as chairman of the Department of Immunology.

INVESTIGATORS' REPORTS

Life and Death During Infection: The Impact of Genes

B. Beutler, C. Arnold, M. Barnes, M. Berger, A. Blasius, B. Croker, K. Crozat, C. Domingo, X. Du, C. Eidenschenk, N. Gnauck, E. Hanley, K. Hoebe, M. Kastner, K. Khovananth, P. Krebs, B. Layton, M. Ioresco, N. Nelson, B. Ortiz, X.-H. Li, E. She, S. Sovath, O. Siggs, K. Whitley, Y. Xia, N. Xiao

During the past 6 years, we have used a classical genetic strategy to understand immunity. We have approached complex phenomena, but not with hypotheses as many biologists do. Instead, we have sought to detectably alter these phenomena by random germ-line mutagenesis and to identify phenotypes via extensive screening. We then identify the causal mutations by positional cloning. In this manner, without any preconceptions about how a system operates, we are able to find the key molecular components required for its function.

Our foremost interest is innate immunity: the inherited system of defense that protects the host against almost all microbes that exist. In one form or another, all multicellular organisms have innate immunity, and many innate immune mechanisms have been conserved through millions of years of evolution. For example, strong similarities exist between the molecules and pathways that mediate innate immunity in insects and in mammals. In some of our screens, which are probes for different, tightly defined aspects of innate immunity, we think that we have found 30% of the components required for normal function.

So far, we have mapped 95 phenotypes to chromosomal intervals and have found 77 of them by using positional cloning. A total of 52 phenotypes of immunologic dysfunction have been mapped, and many more are in the queue for mapping. Most, but not all, of these phenotypes have been found via positional cloning. Some mutations provided new insight into how the innate immune system functions. Others told us something new about how autoimmune processes can occur and can potentially be mitigated.

As sequencing methods improve, perhaps someday a phenotype will be detected in the morning and the causal mutation will be found by afternoon. But affordable whole-genome sequencing remains out of reach, and until it becomes a reality, we must work to perfect achievable methods required to produce and identify phenotypes and to track down the mutations that cause the phenotypes. Toward that end, we have taken some important steps and have developed some exceedingly powerful tools for the fixation and identification of mutations that cause phenotype.

During the past year, we reached an important milestone with the development of an integrated system for finding mutations. In this system, software that interfaces with a robot and a DNA sequencer is used to make finding mutations highly automated. When a mutation is mapped to a critical region, the location is known within specific limits on a certain chromosome. However, without extensive mapping, hundreds of genes may occupy the interval, and all are considered candidates. The software program automatically designs primers for the amplification of exons and splice junctions within which mutations causing phenotype almost always reside. The program then directs a robot to amplify and sequence the exons from both normal and mutant mice, measuring the coverage that is achieved and repeating poorly covered areas in an iterative manner. Finally, it analyzes sequence data and declares the mutation when it is found. Using this tool, we hope to find as many as 50 to 100 mutations each year; previously only 20 or fewer were found.

Another impediment to finding mutations has also been surmounted with the introduction of state-of-the-art assisted reproductive technology. Until now, when a mutation caused susceptibility to a particular viral pathogen, retrieving the mutation has been difficult. The same is true for any conditionally lethal phenotype; as soon as the phenotype is spotted, it is gone. Making the mutation homozygous (a prerequisite for mapping and identification) previously required costly and protracted inbreeding. We are now using a technique called intracytoplasmic sperm injection that permits us to force homozygosity of mutations of this type in a short time in a direct approach. We also use germline cryopreservation, produce mice from embryonic stem cell lines, and create transgenic animals, which are often required to prove the function of a gene.

These technologic improvements have enabled us to accelerate our work substantially, and in the coming year we expect to find many more of the proteins essential for signaling by Toll-like receptors, resistance to viruses, and natural killer cell function. We have also found that some genes can be mutated to yield a

"stronger" host, one that is more resistant to disease. Hence, the mammalian host has not exploited all the mechanisms that it might potentially use to defend itself against infection. The mutations that confer resistance are of great interest to us, because some of them may point to new targets for "host-oriented" chemotherapy for viral infections.

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Regulation of Cell Function by Rho GTPases

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Rho GTPases control the assembly of the actin and microtubule cytoskeletons, the production of reactive oxygen species (ROS), 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, inflammatory responses, and development. We use cellular, molecular, biophysical, and biochemical approaches to understand how the activities of Rho GTPases are regulated, to identify the proteins they interact with to control cell function, and to investigate how these regulatory processes are abnormal in various disease states.

RHO GTPASES AND INNATE IMMUNE FUNCTIONS OF HUMAN LEUKOCYTES

We previously established that the GTPase Rac2 regulates the formation of ROS that are used by human phagocytic leukocytes for microbial killing and that result in inflammatory responses. Our discovery of a functional interaction between Rac2 and cytochrome *b*, a component of the membrane-bound NADPH oxidase, independent of $p67^{phox}$, led us to propose a 2-step mechanism for regulation of electron transfer to form superoxide (Fig. 1). We are mapping the binding site for Rac2 on cytochrome *b* to investigate the molecular basis for regulation of ROS production by Rac2. In addition to their role in innate immunity, NADPH oxidases participate in intracellular signaling. Regulation of nonphagocyte NADPH oxidases is largely not understood, but we are investigating modulation of these enzymes by kinase pathways that phosphorylate regulatory components of the oxidases. A cell-based high-throughput screen for novel regulators is under way.

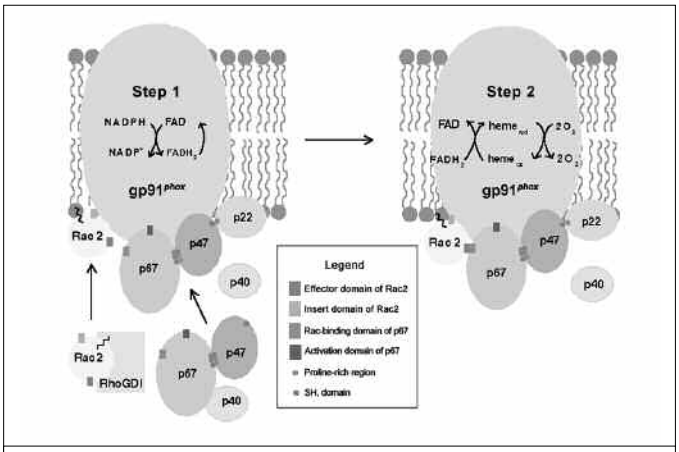

Fig. 1. Two-step activation mechanism for Rac GTPase–mediated regulation of oxidant formation by the phagocyte NADPH oxidase.

We are using live-cell imaging in combination with fluorescent methods to determine the spatial and temporal localization of Rho GTPase activation. We are beginning to determine the molecular signals that govern the chemotactic responses of human leukocytes. Recently, we described the ability of Rac1 signaling in neutrophils to stimulate RhoA activation at the cell rear. Such Rho GTPase cross talk promotes the development of the stable cell polarity necessary to maintain directionality of chemotaxis during inflammatory responses. Studies of the dynamics of Cdc42 activation during neutrophil chemotaxis are ongoing.

REGULATION OF INNATE IMMUNITY BY ANTHRAX TOXINS

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 the function of human leukocytes. We have established that anthrax edema toxin and lethal toxin effectively block the ability of chemoattractant receptors to stimulate the production of ROS by human neutrophils. The molecular basis for such inhibition is under investigation. A requirement for Rho GTPases in the uptake and action of anthrax toxins in macrophages is also under study (Fig. 2).

CYTOSKELETAL REGULATION BY RHO GTPASES

The p21-activated kinases (PAKs) are Rac and Cdc42 effectors that serve as important mediators of chemotaxis, wound healing, tumor metastasis, neurite outgrowth, antigen presentation, and other processes dependent on cytoskeletal polarization. In collaborative studies with G. Danuser and C. Waterman-Storer, Department of Cell Biology, we are using quantitative fluores-

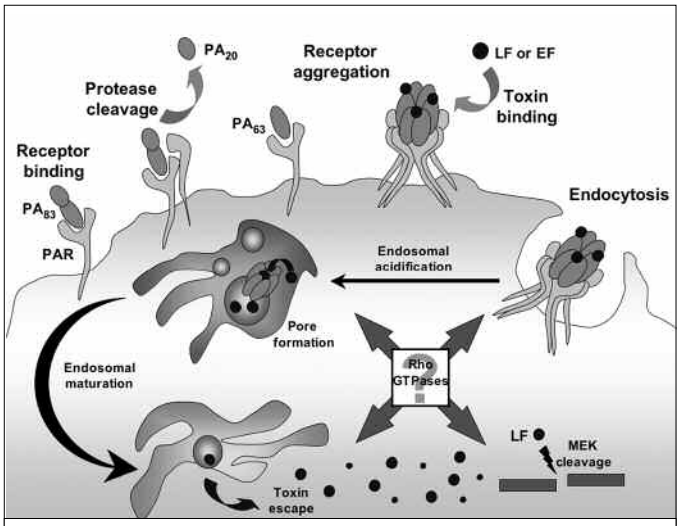

Fig. 2. Rho GTPase regulation of the action of anthrax lethal toxin. Anthrax toxin is a ternary complex consisting of 1 binding subunit, protective antigen (PA), and 2 enzymatic subunits, edema factor (EF) or lethal factor (LF). Full-length PA (PA_{83}) binds to receptors on the cell surface and is cleaved by a furinlike protease to its active form (PA_{63}). Active PA oligimerizes, driving receptor aggregation and internalization by endocytosis. During normal maturation and acidification of the endosomes, PA forms a channel through which EF and LF are transported from the endosomal compartment and into the cytoplasm to act on their respective effectors. Rho GTPases may act to regulate endocytosis, endosomal maturation, and toxin escape or activity. Figure courtesy of Aimee DeCathelineau.

cent speckle microscopy to investigate the regulation of leading-edge actin dynamics by PAK1 downstream of Rac GTPase. We 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. Using a biochemical screen, we identified a unique cofilin phosphatase, termed chronophin, that regulates stimulus-dependent activation of cofilin. Using small interfering RNA to reduce expression of chronophin, we discovered that this phosphatase is involved in the control of cytokinesis during cell division. Chronophin is implicated in the formation of aneuploid cancers; it is overexpressed in such tumors and is an autoantigen in patients with cancer. 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 cytoskeletal changes initiated during cellular energy (ATP) depletion induced by processes such as ischemia.

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 GDI to promote cell transformation and metastasis. We are using imaging methods to provide a quantitative understanding of Rho GTPase–Rho GDI cycle regulation in intact cells.

Cell division also requires highly regulated actin-myosin-microtubule dynamics. We 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 serves as a link between mitotic spindle microtubules and the initiation of Rho-dependent formation of cleavage furrows in dividing cells (Fig. 3). GEF-H1 activity is also

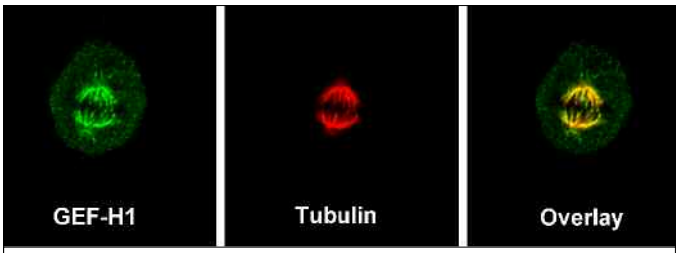

Fig. 3. Immunofluorescent images show colocalization of endogenous GEF-H1 with microtubules (tubulin) in the mitotic spindle.

controlled by cell cycle–dependent kinases. Detailed analysis of the function of GEF-H1 in cell division and motility is under way. Of interest, GEF-H1 is abundant in blood cells and is downregulated by recently developed drugs that inhibit chronic leukemias.

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Human Antibodies and Design of a Vaccine to HIV

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

Among the first questions we 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, 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. Dwek, Oxford Glycobiology Institute, 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 generating human monoclonal antibodies by using not only phage display but also yeast display and the rescue of memory B cells.

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Antibodies and Emerging Viruses

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We are interested in determining the immunogenicity of soluble vs surface glycoproteins of Ebola virus, a filovirus that is one of the deadliest human pathogens. Results indicate strong cross-reactivity and immunogenicity of the different glycoproteins. This finding supports the hypothesis that some of the soluble forms of the glycoproteins act as decoys.

We have also been investigating the effects of a human monoclonal antibody that neutralizes Ebola virus; the antibody was isolated from a patient who was infected with the virus in the Democratic Republic of Congo and who recovered. Previously, we showed that the antibody protects guinea pigs against challenge with Ebola virus. However, studies done in collaboration with scientists at the U.S. Army Medical Research Institute of Infectious Diseases indicate that the antibody does not protect monkeys and indeed appears to offer little benefit even when given at high doses. Surprisingly, virus replication apparently can proceed unhindered in the tissues of the monkeys even in the presence of high serum concentrations of antibody. We are currently attempting to understand this phenomenon and reconcile it with the ability of certain vaccines to protect against challenge with Ebola virus.

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Generation of Lipid-Free Apolipoprotein AI From High-Density Lipoprotein by Macrophage Transfer Proteins

L.K. Curtiss, D.T. Valenta, N.J. Hime, C. Flood, M.R. Richards, R.J. Petrovan, T.A. Bell

The hallmark of an atherosclerotic lesion is the lipid-engorged macrophage or foam cell. Through the accumulation of lipids and the subsequent expression of cytokines and chemokines, macrophages play an obligate role in the progression of atherosclerotic disease. High plasma levels of high-density lipoprotein (HDL) containing apolipoprotein AI protect against atherosclerosis. This HDL is protective because it participates in the transport of excess cellular cholesterol from lesions to the liver for catabolism into bile acids and removal from the body. An initial step in this reverse cholesterol transport is the movement of unesterified cholesterol from peripheral cells (e.g., macrophage foam cells within lesions) to HDL. This transfer occurs in the interstitial space of the intima of a vessel wall and is promoted by the interaction of lipid-free apolipoprotein AI with cellular membrane ATP-binding cassette A1 cellular transporters. Because HDL does not interact with these transporters and apolipoprotein AI is not synthesized by macrophages, this apolipoprotein AI must be released from HDL.

We propose that apolipoprotein AI is derived from HDL by remodeling events accomplished by proteins secreted locally from the cholesteryl ester–loaded macrophages within atherosclerotic lesions. These proteins include the lipid transfer proteins phospholipid transfer protein (PLTP) and cholesteryl ester transfer protein (CETP) and the triglyceride hydrolase hepatic lipase.

We examined the combined effect of CETP and bone marrow–derived hepatic lipase on atherosclerosis in hypercholesterolemic mice deficient in receptors for low-density lipoprotein ($LDLr^{-/-}$ mice). CETP transfers cholesterol from HDL to triglyceride-rich lipoproteins in exchange for triglyceride. Hepatic lipase hydrolyzes both triglycerides and phospholipids in HDL (particularly triglyceride-enriched HDL). The combined actions of CETP and hepatic lipase in vitro remodel HDL and generate lipid-free apolipoprotein AI. Because mice do not express CETP, we examined the effect of a deficiency of

bone marrow–derived hepatic lipase on atherosclerosis in mice that do or do not express a human CETP transgene.

We used transplantation of bone marrow cells that did or did not express hepatic lipase to generate 4 groups of chimeric mice. A deficiency of bone marrow–derived hepatic lipase does not affect the extent of atherosclerosis, either in the presence or absence of systemic CETP activity. However, when the relationship between plasma HDL-cholesterol levels and the extent of atherosclerosis is assessed, an inverse relationship is consistently observed in LDLr$^{-/-}$ mice that express the human CETP transgene. LDLr$^{-/-}$ mice expressing the transgene that lack hepatic lipase because their bone marrow does not express the lipase had extremely low plasma levels of HDL-cholesterol that are highly predictive of increased disease. Lipid-poor apolipoprotein AI is increased when CETP is present and bone marrow cell–derived hepatic lipase is lacking, suggesting that bone marrow cell–derived hepatic lipase participates in the lipidation of apolipoprotein AI in the vessel wall. Thus, macrophage-derived hepatic lipase has antiatherosclerotic potential, but only when CETP is present.

Elevated plasma levels of PLTP are another risk factor for coronary heart disease in both mice and humans. Overexpression of plasma PLTP activity in mice decreases plasma levels of HDL cholesterol. In hypercholesterolemic LDLr$^{-/-}$ mice, systemic PLTP expression increases lesion development with a concomitant decrease in the levels of HDL cholesterol and apolipoprotein AI. However, PLTP expression by macrophages significantly reduces atherosclerosis in LDLr$^{-/-}$ mice. Such complex and sometimes contradictory characteristics of PLTP are primarily attributed to its role in lipoprotein remodeling. Lipid transfer by PLTP in vitro converts triglyceride-rich, spherical HDL into large and small particles with a concomitant release of the lipid-poor apolipoprotein AI. PLTP expression in peripheral tissues alters lipoprotein metabolism, even when liver expression and plasma activity remain unchanged.

We examined the specific role of macrophage PLTP expression by using bone marrow transplantation; we found that macrophage-derived PLTP is atheroprotective in LDLr$^{-/-}$ recipient mice that also express systemic PLTP. This finding suggests that the influence of PLTP on atherogenesis is highly dependent on the protein's site of expression.

We extended these observations by examining the effect of macrophage-derived PLTP on lipoprotein metabolism and the development of atherosclerotic lesions in hypercholesterolemic LDLr$^{-/-}$ mice that also lacked systemic PLTP. We transplanted bone marrow that was or was not deficient in PLTP to double-mutant PLTP$^{-/-}$ LDLr$^{-/-}$ mice. Compared with PLTP-deficient chimeras, chimeras with PLTP-expressing bone marrow had a 62.8% reduction in en face aorta lesions and a 48.0% reduction in heart sinus valve lesions. Thus, macrophage-derived PLTP significantly reduces progression of atherosclerosis in mice that lack systemic PLTP.

Further studies are necessary to confirm that macrophage-secreted CETP, PLTP, and hepatic lipase are directly involved in the remodeling of spherical HDL to enhance cholesterol efflux from macrophage foam cells in atherosclerotic lesions.

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Cell Signal Transduction via Protease-Activated Receptor 1 Mediated by Factor Xa Binding to Cell-Surface Annexin 2

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We found that annexin 2 acts as a cell-surface receptor for factor Xa on human endothelial cells. Binding of factor Xa to annexin 2 on the cell surface selectively activates protease-activated receptor 1; bound annexin cannot mediate the established coagulation protease cascade function of factor Xa. We characterized and confirmed the specificity of the interaction between annexin 2 and factor Xa by showing that other annexin 2–binding ligands, that is, plasminogen, angiostatin, Gla-domain proteins, factor X, factor IX, prothrombin, and ε-aminocaproic acid, inhibit coprecipitation of factor Xa bound to annexin 2.

Expression of tissue factor abolished this novel annexin 2–mediated factor Xa signaling and dimin-

ished binding of factor Xa to annexin 2 on the cell surface. In further studies, we found that annexin 2 regulates factor Xa–mediated activation of protease-activated receptor 1 and that this regulation is specific to factor Xa generated via the intrinsic coagulation pathway; factor Xa–driven signaling mediated by extrinsic tissue factor abrogates the effect of annexin 2.

We propose that annexin 2 regulates factor Xa signaling specifically in the absence of cell-surface tissue factor and may thus have physiologic and even pathologic roles when factor Xa is generated by the intrinsic coagulation pathway.

Endothelial Surface Proteome Diversity in the Tumor Microenvironment

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The gene expression pattern of the vascular endothelium is profoundly influenced by the local microenvironment. Previously, we found 340 differences in transcriptional and organizational differences between endothelial cells of the tumor microvasculature and endothelial cells elsewhere in the same cell donor. We discovered that a unique molecular complex containing neuropilin-1 is expressed in many sites of active angiogenesis or where neuronal guidance is required. However, in the tumor microenvironment, neuropilin-1 is modified by chondroitin sulfate on serine at position 216. This modified neuropilin markedly enhances interaction with the heparin-binding domain (HBD) of vascular endothelial growth factor (VEGF) and associates with the receptor for VEGF, resulting in a novel trimolecular complex consisting of neuropilin-1, chondroitin sulfate, and the VEGF receptor that is found only in the tumor microvasculature. The chemically synthesized 24 amino acid HBD (HBDt) binds with marked affinity to this trimolecular complex.

We successfully targeted this complex in vivo with fusion proteins that incorporate HBDt at the N terminus of selected effector molecules. Previously, we targeted the extracellular domain of tissue factor, inducing highly selective and localized thrombosis, which produces ischemic infarction and eradication of tumors. These fusion proteins are designated selective tumor vascular thrombogens. The selective tumor vascular thrombogen containing HBDt localizes to selected tumor neoangiogenic endothelial surfaces and is docked functionally to appropriate substrate-rich plasmalemma microdomains to locally thrombose the tumor vasculature.

We have now produced a recombinant protein with $VEGF_{165}$ HBDt (cysteine 137–cysteine 160) fused to the Fc of the heavy chain of human IgG1. The resultant protein is a homodimer of 57 kD containing 2 HBDt, which is expressed in human embryonic renal epithelial HEK293 cells. This HBDt-Fc fusion protein has greater HBDt avidity for its target and a longer half-life in the circulation than does HBDt alone. When radiolabeled HBDt-Fc was infused intravenously, the vascular density in the highly vascular N202 mammary tumor model was markedly reduced, leading to tumor regression. Thus, the potential of these HBDt-guided molecular constructs to cause eradication of tumor cells is promising.

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

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A main focus of our laboratory is the molecular analysis of factors that influence the composition of the antibody repertoire and elucidation of the mechanisms that control the V(D)J rearrangement process. In each precursor B lymphocyte, a different set of V, D, and J genes recombine to form exons for the light and heavy chains of the antibody molecule. Each locus has many V, D, and J genes, but the gene segments are not used equally. One of our goals is to understand the basis of this nonrandom use of gene segments.

We previously showed that much of this bias occurs because V genes undergo recombination with different intrinsic frequencies due to differences in the sequence

of the binding site for the recombinase flanking each gene segment. However, other factors clearly influence recombination frequencies; currently, we are focusing on the role of transcription factors and chromatin modifications in controlling accessibility to V(D)J recombination and recombination frequency.

Genes in loci that are undergoing V(D)J recombination are often associated with histones that are acetylated. We hypothesized that the extent of histone modification affects the frequency of recombination of individual genes, and indeed we observed a positive correlation between the relative rearrangement frequency of individual genes in vivo and the extent of acetylation of histones associated with those genes, as assessed by chromatin immunoprecipitation. We are now identifying other histone modifications that likely play a role in regulating gene use in the antibody repertoire.

We have also uncovered a novel role for the transcription factor Pax5 in promoting V(D)J rearrangement. Pax5 is essential for B-cell development. In the absence of Pax5, V_H-to-DJ_H rearrangement is severely impaired. We found Pax5 binding sites in the coding regions of many V_H genes. Furthermore, in collaboration with Z. Zhang and M. Cooper, University of Alabama, Birmingham, we showed that Pax5 binds to the recombinase proteins RAG1 and RAG2. Hence, we propose that Pax5 may recruit RAG1 or RAG2 to the recombination signal sequence or may stabilize the interaction of the RAG complex with its binding site. We are investigating other roles of Pax5 in shaping the antibody repertoire.

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 during fetal and neonatal life lack an enzyme, terminal deoxynucleotidyl transferase; this enzyme 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 compartment, suggesting that the fetal/neonatal repertoire of antibodies, which is quite different from that generated in adults, may be particularly useful against blood-borne pathogens.

In other studies, we are examining the breakdown of B-cell tolerance in autoimmunity. When precursor B cells successfully recombine both heavy- and light-chain gene segments, they express a B-cell receptor for the first time. If the receptor is autoreactive, then the immature B cell normally continues to undergo light-chain V-J rearrangement until an innocuous receptor is made. This process is termed receptor editing and is an important checkpoint in B-cell tolerance. We have evidence that this process is not functioning as efficiently in lupus-prone mice as in nonautoimmune mice, and we are investigating why this difference occurs. 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.

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Development of Anti-HIV Microbicides

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Each day, more than 14,000 new HIV infections occur, 50% of them in women. In regions where the primary mode of HIV transmission is heterosexual contact, the number of women with HIV infections or AIDS has exceeded 50% of the total number of people infected. These numbers highlight the impact of the HIV pandemic on women.

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 high endogenous error rate of the HIV reverse transcriptase drives the development of resistance and genomic diversity in HIV. The current pan-resistance 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 2 enzymes. This concern may be extended to inhibitors

of HIV entry because of the rapid emergence of mutations in the HIV envelope proteins gp120 and gp41 under selective pressure. Thus, identification of host proteins (rather than viral proteins) critical for HIV infection is imperative for the development of novel 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. Interactions between HIV and syndecan-3 occur via the V3 loop of gp120 and the heparan sulfate chains of syndecan-3. Thus, HIV exploits syndecan-3 to mediate HIV transmission, and an effective microbicide should target syndecan-3 on dendritic cells to prevent transmission.

We identified a short peptide as a potent inhibitor of HIV. Interestingly, the peptide corresponds to the N terminus of the membrane-associated nonstructural protein 5A (NS5A) of hepatitis C virus, an essential component of the viral replication complex. NS5A peptide blocks HIV infection before cell entry, by targeting components of the viral membrane other than gp120/gp41. Our data suggest that the NS5A peptide blocks HIV infection by rupturing the viral membrane by interacting with a receptor enriched in the HIV membrane. The rupture is specific; the peptide does not affect the integrity of cellular membranes or the membranes of other enveloped viruses. We are conducting in vitro and in vivo studies to support the preclinical development of the hepatitis C virus NS5A peptide as a novel first-in-class anti-HIV microbicide.

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Modulation of HIV and Hepatitis C Virus Infections by Intracellular Tripartite Motif 5 Protein and Cyclophilins

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The mechanisms by which the tripartite motif protein TRIM5 decreases HIV infection by acting on incoming viral capsid cores are unknown. We found that TRIM5 binds directly and specifically to HIV cores and forms high molecular weight complexes. We also found that TRIM5 does not accelerate uncoating of capsid cores, but rather accelerates degradation of capsid molecules. We are investigating the possibility that the formation of complexes consisting of TRIM5 and capsid cores directs incoming capsid cores into abortive compartments where capsid molecules are degraded.

Although it is well documented that cyclosporine A inhibits HIV infection by binding to cyclophilin A, recent studies suggest that the drug also inhibits hepatitis C virus (HCV) infection in vitro by acting on cyclophilin B rather than on cyclophilin A. In a clinical study, we found that a cyclosporine A analog only weakly decreased HIV viral load (0.7 $\log_{10}$), whereas it profoundly decreased HCV viral load (3.6 $\log_{10}$). In patients treated with the cyclosporine A analog, the intracellular levels of cyclophilin B, but not of cyclophilin A, dramatically decreased. The decrease in cyclophilin B coincided with the decrease in HCV viral load. These data are the first from studies in humans that support the hypothesis that cyclophilin B plays an important role in HCV replication. The results also indicate that cyclophilin B is a valid target for the development of anti-HCV drugs.

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Molecular Interactions in T-Cell Development and Activation

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FLUORESCENCE RESONANCE ENERGY TRANSFER OF INTERACTIONS BETWEEN T-CELL RECEPTORS AND CORECEPTORS IN T-CELL ACTIVATION

Forster or fluorescence resonance energy transfer (FRET) is a physical phenomenon that 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.

Using FRET, we showed that the coreceptor CD8 and the T-cell receptor (TCR) signal-transducing protein CD3ζ are recruited to the "immunologic synapse," where they interact when antigenic MHC-peptide complexes are presented to the T cell. No FRET occurs when weaker (e.g., TCR antagonist) ligands are used. We discovered previously that endogenous MHC-peptide complexes aid in recognizing antigenic MHC-peptide complexes. The interaction between CD8 and the endogenous MHC-peptides improves TCR recognition of the antigenic MHC-peptides, including the ability to associate with CD8. This surprising finding suggests how T cells can find the "needle" (small amounts) of antigen in the "haystack" of nonstimulatory MHC-peptides.

We are now testing whether the TCR itself must interact with the endogenous MHC-peptide or whether the CD8 interaction with the MHC molecule is sufficient to provide the aid for antigenic recognition. Of the more than 15 different endogenous peptides that we have tested, all aid in recognition of antigen, even of weak agonists, a finding that suggests that TCR recognition of endogenous MHC-peptide is not required for the activity of the endogenous peptides, but a more rigorous test is in progress. We have also compared TCR-CD8 interactions induced by peptides straddling the border of positive and negative thymic selection. We found that compared with induction by negative selecting ligands, the induction of FRET by positive selecting ligands is delayed. We are directly testing the potentially different roles of CD8αα and CD8αβ in formation of the immunologic synapse and the dynamics of association of the kinase Lck with CD8 before and during antigenic stimulation.

PROTEIN KINASE C η IN THYMOCYTE DEVELOPMENT

We previously showed that the η isoform of protein kinase C (PKC) is upregulated during positive selection of developing thymocytes. Of the PKC isoforms, only PKCθ is known 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 PKCθ have normal thymic selection suggested that PKCη could be replacing PKCθ in the developing thymocytes.

We found that PKCη is also naturally recruited to the synapse in mature thymocytes and T cells. In the absence of PKCθ, PKCη is expressed at an earlier stage of thymocyte development, where it functions in place of PKCθ. Inhibition of PKCη expression by short hairpin RNAs during thymocyte development results in inhibition of T-cell development. To study the role of PKCη in T-cell development and activation, we have produced mice that lack PKCη under certain conditions.

A NOVEL PROTEIN IMPORTANT IN T-CELL DIFFERENTIATION

We identified a novel protein that is expressed primarily during thymocyte differentiation, particularly in immature, pre–positive selection thymocytes. We have produced a strain of mice that lack the gene for this protein. Mice that lack the gene have defects in thymic positive selection and in the ability to be stimulated through the TCR. Thus, this protein has an important role in T-cell signaling and development. We are determining its interaction partners in the T-cell activation cascade.

TCR ENDOCYTOSIS, RECYCLING, AND UBIQUITINATION

Because allelic exclusion of the TCR α-chain is maintained after translation, many mature T cells express 2 α-chain proteins. However, expression of 2 α-chains on the cell surface is rare. We previously showed that 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 α-chains on the cell surface. We are also using FRET between ubiquitin monomers and TCR subunits labeled with fluorescent proteins to analyze ubiquitination of TCRs after endocytosis.

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Functional Roles of Novel Rho Guanine Nucleotide Exchange Factors in B Lymphocytes

A.L. Gavin

Rho guanine nucleotide exchange factors (GEFs) are important activators of Rho GTPases. In mammals, the Ras homology (Rho) subfamily of monomeric GTPases, including Rac1-3, RhoA-G, 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.

In expression studies, my colleagues and I identified a novel putative Rho GEF, named Fgd2, that was downregulated after IgM stimulation by either immature or mature B cells. Fgd2, highly expressed in B cells and professional antigen-presenting cells, is a GEF for the Rho GTPases and has marked sequence identity with known Cdc42 GEFs. Another related family member, Fgd3, is also expressed in leukocytes, including B cells, T cells, and natural killer cells.

Fgd2 and Fgd3 belong to a gene family identified by relation to the originally discovered member Fgd1, the gene responsible for a faciogenital dysplasia called Aarskog-Scott syndrome. Although all members of the gene family consist of conserved functional motifs, significant differences at the N termini may play unique functional roles for each member (Fig. 1). Like many other Rho GEFs, the Fgd family contains a Rho GEF domain, the so-called dbl homology domain; a pleckstrin homology (PH) domain; a FYVE domain; and a second

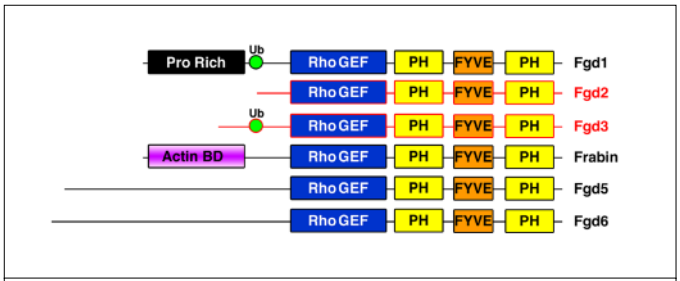

Fig. 1. Protein domains of Fgd family members. Ub, ubiquitinylation site; Pro Rich, proline rich; Actin BD, actin-binding domain.

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.

We have produced animals deficient in Fgd2 and will produce other animals deficient in Fgd3. Because Fgd2 and Fgd3 are the only Fgd members specifically expressed in cells of the immune system, our goal is to investigate the role these genes play in B-cell signaling and function.

Signaling Pathways in the Innate Immune System

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The innate immune response is triggered by interaction of microbial pathogens and inflammatory cytokines with their respective receptors. We focus on intracellular pathways that conduct signaling from Toll-like receptors (TLRs) and cytokine receptors, leading to induction or modulation of gene expression that promotes inflammation in a broad sense. A pivotal role in transducing inflammatory signals is played by the p38 MAP kinase pathway, which is one of our primary research interests. We are also investigating

the involvement of microRNAs in posttranscriptional regulation of inflammatory gene expression.

ACTIVATION OF P38α MAP KINASE

The primary activation pathway of p38α MAP kinase is a trikinase cascade that consists of p38α MAP kinase, MAP kinase kinase (MKK), and MKK kinase (MAP3K). However, autoactivation of p38α MAP kinase can also occur and is promoted by interaction with transforming growth factor β–activated protein kinase 1 binding protein 1 (TAB1). MKK3 and MKK6 can activate p38α, and MKK4 can assist in p38α activation through activation of Jun N-terminal kinase. We set out to investigate whether a given stimulus activates a unique mechanism or whether these mechanisms make contributions parallel to the activation of p38α.

Studies with cells deficient in MKK3/6 and MKK4/7 support the idea that TAB1 can activate p38α independent of MKKs. However, MKK-independent p38α phosphorylation accounts for only a small part of total p38α phosphorylation, depending on the nature of the stimulus. Surprisingly, TAB1 can also inhibit p38α phosphorylation induced by peroxynitrite. This inhibitory effect most likely is not mediated by MAP kinase phosphatase-1, but by an unidentified binding factor.

In conclusion, the trikinase cascade is the most important p38α phosphorylation pathway, although multiple pathways can be activated in a cell type– and stimulus-dependent way. The presence of multiple pathways of p38α activation may allow for differential responses to a variety of environmental clues with the desired specificity and strength.

THE P38β PATHWAY IN REPLICATION OF *DROSOPHILA* C VIRUS

We previously reported that in *Drosophila*, lack of p38 isoforms leads to an increased susceptibility to bacterial infection, thus implicating p38 in anti-infectious mechanisms. Ongoing investigations have now revealed that a lack of p38β impairs replication of the picorna-like *Drosophila* C virus (DCV) by decreasing the translational efficiency of viral mRNAs. This decreased translation increased the survival rate 3-fold. Similar results were obtained by inhibiting p38 in wild-type flies.

Flies deficient in LK6, a downstream target of p38 homologous to mammalian MAP kinase signal–integrating kinases 1 and 2, have the same phenotype as flies deficient in p38β. This finding suggests that LK6 is the downstream target of p38β required for DCV replication. LK6 reportedly phosphorylates the cap-binding eukaryotic initiation factor 4E, leading to increased translation of capped messengers. However, DCV mRNAs are translated in a cap-independent fashion, and replication of DCV in p38β- or LK6-deficient flies cannot be rescued by overexpression of constitutively active eukaryotic initiation factor 4E. Thus, LK6 probably also targets other components of the translational machinery. We are investigating this possibility.

4-1BBL AND SUSTAINED LIPOPOLYSACCHARIDE-INDUCED TNF PRODUCTION IN MACROPHAGES

Upon exposure to lipopolysaccharide, macrophages rapidly start the production of TNF, which can last for up to a day. The initial production is mediated via interactions between TLR4 and the adaptor proteins MyD88 and TRIF, which lead to the transient activation of the transcription factor NF-κB and transcription of TNF mRNA. Sustained production is mediated via MyD88/TRIF– and NF-κB–independent pathways and relies on the interaction of 4-1BBL with TLR4. 4-1BBL is a transmembrane molecule that is rapidly induced after TLR signaling and that interacts with cell-surface TLR4. This interaction gives rise to late-phase activation of CREB and CREB-binding protein; activation of p38, Jun N-terminal kinase, and extracellular signal–regulated kinase; and stabilization of the TNF mRNA, all together leading to the sustained production of TNF.

Thus, distinct signaling pathways are involved in initial and sustained TNF production in macrophages. We plan to identify the signaling adaptor of 4-1BBL and elucidate in more detail the mechanism by which 4-1BBL induces the expression of inflammatory mediators.

MICRORNAS IN ANTIVIRAL RESPONSES

MicroRNAs are single-stranded RNA molecules of 19–23 nucleotides that originate from longer and imperfectly matching hairpin precursors processed by several ribonucleoprotein complexes. The cytosolic enzyme Dicer converts hairpin precursors into 19- to 23-nucleotide RNA duplexes and therefore is of key importance for the maturation of microRNAs.

We generated Dicer-deficient mice and investigated macrophage sensitivity to infection with vesicular stomatitis virus (VSV). Compared with macrophages from wild-type mice, macrophages from Dicer-deficient mice had increased VSV replication. Furthermore, we found that the anti-VSV functions of Dicer are at least partly mediated by microRNA-24 and microRNA-93, which target viral RNA genes that encode the RNA-dependent RNA polymerase complex. We confirmed the involvement of the microRNAs in the antiviral response by using VSV with mutations in the predicted microRNA

target sites. In wild-type mice, mutated VSV was more pathogenic than wild-type VSV, emphasizing the role of microRNAs in the innate antiviral response. MicroRNAs may play an important role in the innate defense against viral infection in mammals.

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Specificity and Function of Intraepithelial γδ T Cells

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We have a long-term interest in interactions between intraepithelial γδ 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 γδ T cells. Our results have defined unique properties of these cells and support a specialized role for intraepithelial γδ T cells in immune surveillance, wound repair, inflammation, and protection from malignant tumors.

IDENTIFICATION OF MOLECULES REQUIRED FOR γδ T-CELL ACTIVATION

In murine skin, γδ T cells express an invariant γδ T-cell receptor that recognizes an unknown antigen expressed by damaged or malignant neighboring keratinocytes. We have produced soluble skin γδ 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 γδ T cells by binding to coreceptors or costimulatory molecules on the T-cell surface. Skin γδ T cells do not express classical molecules, including CD4, CD8, and CD28, known to affect activation of γδ T cells.

We recently identified several molecules expressed by the skin γδ T cells and keratinocytes that provide important costimulatory signals for activation of γδ T cells. One such molecule, JAML, is uniquely costimulatory for epithelial γδ T cells. We have identified another JAM family member, the coxsackievirus-adenovirus receptor, as a ligand for JAML that is expressed on epithelial cells in the skin and intestine. Structural studies, performed by our collaborators I.A. Wilson and colleagues, Department of Molecular Biology, indicate a novel signaling model for γδ T-cell activation. Interactions between JAML and the coxsackievirus-adenovirus receptor may play important roles in γδ T-cell responses during wound repair and other epithelial challenges.

We also found that the semaphorin Sema4D (CD100) is expressed by skin and intestinal γδ T cells upon activation. CD100 binds to a member of the plexin superfamily of semaphorin receptors, plexin-B2, expressed on epithelial cells. We found that interactions between CD100 and plexin-B2 deliver key signals both to the γδ T cells and to epithelial cells in the skin and intestine that regulate cell morphology.

A ROLE FOR INTRAEPITHELIAL γδ T CELLS IN EPITHELIAL TISSUE REPAIR

We found that skin γδ T cells have a role in the reepithelialization stage of wound repair. The γδ T cells are activated at wound sites and produce cytokines,

including keratinocyte growth factors 1 and 2. In the absence of skin γδ T cells, keratinocyte proliferation and tissue reepithelialization after wounding are defective. Recent results indicated that a keratinocyte-responsive γδ T-cell receptor is necessary for activation of the T cells by damaged keratinocytes during wound healing and is also required for the maintenance of T cells in the epidermis. In addition, we found that the skin γδ T cells are necessary for the recruitment of inflammatory cells into the wound site. In a novel mechanism, keratinocyte growth factors produced by γδ T cells stimulate production of hyaluronan by epidermal cells, which then controls migration of macrophages into wounds.

Skin γδ 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 γδ T cells produce this growth factor, which affects wound healing and apoptosis in the skin. Work in progress indicates similar functions for intraepithelial γδ T cells in wound healing in human skin. Together these results indicate a role for skin γδ T cells in multiple aspects of wound repair and for homeostasis of the epithelium.

In previous studies, we showed that intestinal intraepithelial γδ T cells play a similar role in responding to tissue damage in a model of colitis. More 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 γδ T-cell functions in the gut. Similar studies are in progress to determine the roles of intranasal γδ T cells in a model of allergic rhinitis. Results in these models support our hypothesis that intraepithelial γδ T cells respond to epithelial damage or disease and play important roles in tissue repair and epithelial homeostasis. Additional studies should provide information that will further define the role of γδ T cells in epithelial inflammatory disorders and may be useful in designing or testing new therapies.

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Mechanisms of γδ T-Cell Dysfunction in Nonhealing Wounds

J.M. Jameson, R. Mills, K. Taylor

Skin γδ T cells are activated by stressed or damaged keratinocytes to produce molecules such as insulin-like growth factor 1 (IGF-1) and keratinocyte growth factors that are important for the maintenance of skin homeostasis and wound repair. We are examining skin γδ T cells in nonhealing wounds to investigate the mechanisms that inhibit normal wound healing.

CROSS TALK OF γδ T CELLS AND KERATINOCYTES IN THE SKIN IN DIABETES

Treatment of nonhealing wounds is a considerable problem for patients and healthcare services. In murine models of diabetes, decreased levels of IGF-1 and keratinocyte growth factors in wounds contribute to defective wound repair. When these growth factors are replenished, the markedly delayed wound repair is reversed. Previously, we showed that skin γδ 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 γδ T cells. We have identified a defect in the activation state and survival of skin γδ T cells in the skin of diabetic mice.

Currently, we are examining mechanisms that may contribute to skin γδ T-cell dysfunction in diabetes, including changes in insulin, hyperglycemia, and inhibition by glucocorticoids. In addition, the decreased levels of IGF-1 in the epidermal compartment may result in reduced signaling by IGF-1 receptors on skin γδ T cells, a situation that may exacerbate dysfunction of skin γδ T cells.

FUNCTION OF γδ T CELLS IN RESPONSE TO RAPAMYCIN

Rapamycin (sirolimus, Rapamune) is approved by the Food and Drug Administration for prophylaxis of acute rejection of transplanted organs. Patients who receive rapamycin have an increased incidence of complications of wound healing. Because these patients have difficulties with wound healing, rapamycin may not only be targeting allograft-specific αβ T lymphocytes but also be suppressing skin γδ T cells. We have observed

defects in the proliferation of skin γδ T cells and the production of IGF-1 in the presence of rapamycin. We are investigating the mechanism of this suppression.

Once we better understand the role of T cells in tissue repair, it may be possible to design therapies that enhance the ability of these immune cells to heal ulcers and chronic wounds. Our objective is to determine the mechanisms by which skin γδ T cells, normally important in wound repair in healthy wild-type mice, are not functioning properly in chronic wounds.

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Regulators of T-Lymphocyte Development and Function

J. Kaye, P. Aliahmad, M. Fung, O. Garijo, C. Krieg, N. Sanathara

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 a lineage diversification process, termed positive selection, that results in matching T-cell antigen receptor specificity with cellular function. We are interested in the molecular regulation of positive selection. In addition, our identification of a cell-surface protein that is first expressed in the T-cell lineage during positive selection has led to studies on regulation of $CD8^+$ T-cell memory.

REGULATION OF THYMOCYTE SELECTION BY A NUCLEAR ARCHITECTURAL PROTEIN

We identified thymocyte selection–associated high mobility group (HMG) box protein (TOX) several years ago. Members of the HMG box protein superfamily share one or more copies of a sequence-related and structurally related DNA-binding domain that can recognize distorted DNA structures and often modify chromatin by bending DNA. In general, HMG box proteins function as architectural factors that regulate gene expression by promoting formation of transcriptional complexes or by acting as components of chromatin-remodeling complexes.

We found that TOX belongs to a small subfamily of evolutionarily conserved proteins, whose additional members have recently been designated TOX2–TOX4. These proteins share almost identical HMG box sequences. The HMG box domain in TOX can recognize distorted DNA but is a relatively poor bender of DNA, because of the lack of a critical internal wedge residue.

Expression of TOX in the thymus is developmentally regulated. Signaling through the serine/threonine phosphatase calcineurin is required for positive selection of thymocytes and the gene for TOX is a target of this signaling pathway. 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 completion of thymic positive selection that results in nearly complete loss of the $CD4^+$ T-cell lineage. This finding is the first report of a protein involved in regulation of this specific stage of positive selection. Interestingly, loss of TOX also significantly inhibits development of other regulatory T-cell subsets, a finding important for understanding how these distinct cell lineages are formed from common precursors. In addition to defining a role for TOX in the thymus, we are using conditionally TOX-deficient mice to determine whether TOX plays a role in germinal center reactions, a process essential for T cell–dependent humoral immune responses.

NEGATIVE REGULATION OF T-CELL RESPONSES

The functional outcome of engagement of the T-cell antigen receptor is modulated by secondary signals, which can have costimulatory or coinhibitory functions. A number of years ago, we isolated a gene that encodes a cell-surface protein of the immunoglobulin superfamily, now designated B- and T-lymphocyte attenuator (BTLA), that is upregulated during positive selection and that is expressed by mature lymphocytes and antigen-presenting cells. Evidence indicates that this protein can act as a negative regulator of lymphocyte activation. We have produced BTLA-deficient mice and panels of monoclonal antibodies specific for BTLA to analyze the in vivo function of this protein.

One of our monoclonal antibodies acts as an agonist for this inhibitory molecule, thereby inhibiting facets of T-cell activation. In vivo studies indicated that BTLA is a negative regulator of homeostatic expansion of T cells and production of $CD8^+$ memory T cells. For vaccines against intracellular infections and tumors, development of methods to regulate $CD8^+$ T-cell responses and memory formation is paramount, and thus BTLA may be a useful therapeutic target in this regard. Data also indicate that one of our monoclonal antibodies to BTLA prolongs survival of pancreatic islet allografts in mice when used in combination therapy.

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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 primarily 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. 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 established an in vitro reconstitution system for lung epithelium that we use to examine 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 investigating 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 identified 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 colon 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 Nox in health and disease.

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Molecular Mechanisms Regulating Tumor Progression

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THE FOURTH MAP KINASE PATHWAY

Big mitogen-activated kinase 1 (BMK1), also called extracellular signal–regulated kinase 5, a newer member of the mammalian MAP kinase family, is activated by angiogenic growth factors. Using a mouse model in which expression of the gene for BMK1 can be deleted, we showed that the BMK1 pathway is required for tumor-associated angiogenesis and consequent tumor growth through the BMK1/ribosomal S6 kinase/ribosomal protein S6 pathway. To investigate and define the function and molecular actions of the BMK1 pathway in endothelial cells and in angiogenesis, we are investigating the signaling pathways that interact with or are regulated by the BMK1 cascade in endothelial cells and are exploring the mechanism of action of the BMK1 cascade during angiogenesis. These studies should provide new information on the regulatory mechanisms of neovascularization. We hope to use the results to identify novel and important targets for a more effective and specific therapeutic intervention for human cancer by inhibition of tumor-associated angiogenesis.

TUMOR SUPPRESSOR/PROTEIN CHAPERON TID1

Tid1 is the human counterpart of the *Drosophila* tumor suppressor Tid56. Mutations that cause loss of function of the gene for Tid56 result in tumorous imaginal discs due to continuous cell proliferation without differentiation. Using yeast 2-hybrid screening, we found that Tid1 interacts with the signaling domain of the receptor protein-tyrosine kinase ErbB2/Her2. Subsequent studies indicated that increased expression of Tid1 protein in breast cancer cells overexpressing ErbB2 facilitated the ubiquitination and degradation of ErbB2 and caused growth inhibition of the cells. Moreover, using RNA interference to deplete the physiologic levels of Tid1 in breast cancer cells, we discovered that the metastatic potential of Tid1-depleted cells was substantially enhanced. This enhancement was due to increased production of IL-8 through upregulation of the nuclear transcription factor NF-κB in the Tid1-depleted cells.

Thus, because Tid1 attenuates signals generated from the ErbB2 receptor and negatively regulates the activity of NF-κB, we hypothesize that Tid1, like its *Drosophila* counterpart, may be an important tumor suppressor, especially in breast carcinogenesis and metastasis. To evaluate this hypothesis in an animal model, we have established a mouse model in which the gene for Tid1 can be deleted specifically in mammary epithelial cells. We are investigating and evaluating the mechanistic role of Tid1 as a tumor suppressor in the regulation of tumorigenesis and metastasis of breast cancer not only at the molecular and cellular levels but also in an organismal context.

Host-Pathogen Interactions: Mechanisms and Applications

E. Li, S.P. Lad, D. Scott, G.Z. Wang

Microbial pathogens can be classified into 2 broad categories: those that infect the host accidentally and those that do so for growth. The outcome of an infection by "accidental" pathogens is commonly associated with severe host inflammatory responses and is often lethal. In contrast, obligate intracellular pathogens such as chlamydiae and rickettsiae have developed efficient yet poorly defined mechanisms to evade host immune surveillance and secure a favorable habitat. We focus on host responses to viral and bacterial infections. We use infections with adenovirus and the obligate intracellular bacterial pathogen *Chlamydia trachomatis* as models systems.

Chlamydia trachomatis infection affects more than 140 million persons worldwide and is the most preventable cause of blindness and of sexually transmitted diseases. *Chlamydia trachomatis* infection is the most frequently reported sexually transmitted infection in the United States and most industrialized countries, and the infection and the subsequent consequences disproportionately affect women, especially young women, by causing pelvic inflammatory disease and infertility.

Although chronic inflammation is the underlying mechanism of chlamydial diseases, a hallmark of a chlamydial infection is its asymptomatic nature. Most people infected with chlamydiae are not aware of an active infection and hence do not seek a treatment until severe signs and symptoms appear.

We found that *Chlamydia* species can downregulate host inflammatory responses by converting a regulatory molecule of the inflammation pathway to a negative inhibitor of the same pathway. Specifically, chlamydial infection promotes p65/RelA cleavage. The N-terminal cleavage product functions as a dominant negative inhibitor of the NF-κB pathway and hence can block NF-κB–modulated gene expression of inflammatory responses. In further studies, we found that the protease that promotes p65 cleavage is a tail-specific protease.

Chlamydial infections increase the risk for other sexually transmitted infections, such as those caused by herpes simplex viruses, oncogenic papillomavirus, and HIV. In another project, we are focusing on mechanistic studies of chlamydial infection as a risk factor for sexually transmitted infections.

We are also designing and modifying adenovirus for targeted gene delivery. Although widely used for gene therapy studies, adenovirus-based vectors cannot target specific tissues. We generated modified adenoviruses that are equipped with a tumor-targeting antibody, enabling the selective delivery of therapeutic genes to tumor cells by antibody-guided "missiles."

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Suppression of Metastasis, Angiogenesis, and Atherothrombosis

C. Liu, Y. Liu, F. Guo, J. Huang, W. Wu

SUPPRESSION OF METASTASIS AND ANGIOGENESIS BY SELECTIVE ABLATION OF TUMOR-ASSOCIATED MACROPHAGES

Development of both primary and metastatic neoplasms depend on the tumor microenvironment. Tumor growth requires angiogenesis, and the tumor-associated macrophages (TAMs) are key producers of growth factors, such as vascular endothelial growth factor, that induce angiogenesis and support tumor cell survival. Legumain, an asparaginyl endopeptidase, is specifically 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 TAMs and endothelial cells.

Targeting resident cells in the tumor microenvironment has distinctive advantages, because both TAMs and endothelial cells are nontransformed and are much more sensitive to chemotherapeutic agents than are tumor cells, which often are multidrug resistant. The prodrug treatment effectively reduces the number of TAMs in tumors and results in a significant decrease in angiogenic factors and other growth factors that support tumor cell survival. The prodrug suppresses spontaneous metastasis and significantly reduces the number of circulating tumor cells and extends survival of the host without toxic effects. Our findings indicate that TAMs play a critical role in tumor development and metastasis. Selective ablation of TAMs is a novel anticancer strategy that targets multiple steps during tumor metastasis and angiogenesis.

SUPPRESSION OF TISSUE FACTOR–MEDIATED ATHEROTHROMBOSIS

Thrombosis mediated by tissue factor causes most of the acute manifestations of atherosclerosis, such as acute myocardial infarction and stroke. Using in vivo panning of phage display peptide libraries, we identified peptides that selectively bind to atherosclerotic lesions. With these lesion-binding peptides and proteomic methods, we have detected high levels of glu-

cose-regulated protein 78 and $\alpha_3\beta_1$ integrins among other proteins on surfaces of and within atherosclerotic lesions. These results indicate that the endoplasmic reticulum stress responsive pathway (glucose-regulated protein 78) and extensive remodeling (integrins) operate in the microenvironment of atherosclerotic lesions.

Tissue factor pathway inhibitor (TFPI) is a potent inhibitor of tissue factor–mediated coagulation. We constructed targeted TFPIs (tTFPIs) composed of (1) inhibitory Kunitz domains 1 and 2 of TFPI and (2) glucose-regulated protein 78 binding peptide or integrin-binding entities. We found that expression of tTFPIs in cells led to persistent targeted anticoagulant activity that can affect nearby cells through a "bystander effect." Local delivery of tTFPIs to atherosclerotic lesions by viral vectors is a potential therapy for suppressing tissue factor–mediated atherothrombosis.

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Tissue Factor, Coagulation Proteases, and Protease-Activated Receptors in Hemostasis, Thrombosis, Heart Remodeling, and Inflammation

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Tissue factor (TF) is the primary activator of the blood coagulation cascade. Activation of the coagulation cascade generates thrombin, which leads to fibrin deposition and platelet activation. We are interested in the role of TF in hemostasis, thrombosis, and ischemia-reperfusion injury. In addition, we are investigating the role of the phosphatidylinositol-3′-kinase (PI3K)–Akt pathway in lipopolysaccharide-induced coagulation and inflammation.

HEMOSTASIS

The tissue-specific pattern of TF expression suggests that certain tissues, such as heart, brain, or lung, require additional hemostatic protection after vessel injury. For example, the heart may express a large amount of TF because blood vessels, particularly capillaries, are prone to mechanical damage. We have shown that mice expressing low levels of TF (1% of the levels in wild-type mice) have hemostatic defects in many organs, including the heart. We determined that the threshold of TF required to maintain heart hemostasis under normal conditions is about 30% of wild-type levels. In addition, mice with a selective deletion of the TF gene in cardiac myocytes had a mild hemostatic defect that was significantly increased after challenge with isoproterenol. Moreover, cardiac myocyte-specific overexpression of TF abolished the hemostatic defects in low-TF mice. Together, these data indicate that TF expressed by cardiac myocytes plays an important role in heart hemostasis under normal and pathologic conditions.

THROMBOSIS

Increased TF expression within the vasculature induces thrombosis. For instance, during sepsis, lipopolysaccharide derived from gram-negative bacteria leads to disseminated intravascular coagulation. We are interested in the relative contribution of TF expression by different vascular cells to lipopolysaccharide-induced coagulation in a mouse model of endotoxemia. We have produced mice that express wild-type levels of human TF in the absence of mouse TF. We used these mice to investigate the relative contribution of TF expressed by hematopoietic and nonhematopoietic cells to lipopolysaccharide-induced coagulation. Using a bone marrow transplantation strategy in combination with an inhibitory antibody that blocks human but not mouse TF, we found that both hematopoietic and nonhematopoietic cells contribute to lipopolysaccharide-induced coagulation. Furthermore, using mice with a tissue-specific deletion of the TF gene in either myeloid and/or endothelial cells, we showed that both cell types contribute to lipopolysaccharide-induced coagulation. Further studies will determine if deletion of TF from platelets has an impact on lipopolysaccharide-induced coagulation. These data indicate that multiple cell types contribute to lipopolysaccharide-induced coagulation.

HEART REMODELING AFTER ISCHEMIA-REPERFUSION INJURY

Previously, we showed that protease-activated receptor 1 (PAR-1), the cellular receptor for thrombin, contributes to heart remodeling and hypertrophy after ischemia-reperfusion injury without affecting the initial

size of the infarct. Moreover, cardiac myocyte–specific overexpression of PAR-1 leads to cardiac hypertrophy and dilated cardiomyopathy in mice. In vitro studies indicated that activation of PAR-1 or PAR-2 induces similar hypertrophic responses in cardiac myocytes.

We produced mice with a cardiac myocyte–specific overexpression of PAR-2. Preliminary studies indicate that cardiac hypertrophy developed in these mice, as indicated by an increase in the ratio of heart weight to body weight and in expression of markers of hypertrophy. We plan to use PAR-2–deficient mice to investigate the role of PAR-2 in heart remodeling after ischemia-reperfusion injury.

INFLAMMATION

We showed previously that pharmacologic inhibition of the PI3K-Akt pathway inhibits lipopolysaccharide-induced coagulation and inflammation both in vitro and in vivo. We extended these studies by using a genetic approach. We used macrophages from $p85\alpha^{-/-}$ mice, which have reduced PI3K activity, and macrophages from $PTEN^{flox/flox}$/LysMCre mice, which have increased Akt activity. Analysis of lipopolysaccharide signaling in $p85\alpha^{-/-}$ and $PTEN^{-/-}$ macrophages indicated that the PI3K-Akt pathway inhibited activation of MAP kinases but did not affect lipopolysaccharide-induced nuclear translocation of NF-κB. Lipopolysaccharide induction of TNF-α and TF gene expression was increased in $p85\alpha^{-/-}$ macrophages and decreased in $PTEN^{-/-}$ macrophages compared with wild-type macrophages. Importantly, inflammation and coagulation were enhanced in endotoxemic mice given $p85\alpha^{-/-}$ bone marrow. Taken together, our results indicate that the PI3K-Akt pathway negatively regulates lipopolysaccharide signaling and gene expression in monocytes/macrophages.

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Regulating Adaptive Immunity

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We focus on the cellular and molecular mechanisms that regulate adaptive immunity. Our goal is to modify antigen-specific immunity induced by vaccination with proteins to optimize long-term protection through strong and specific immune memory. Generating antigen-specific B-cell memory of high affinity is our major area of research, with emphasis on defining the helper T cell regulation required to control the adaptive response to foreign proteins. Recently,

we extended our studies into the earliest innate immune events that initiate and shape the adaptive response. If we can understand the rules that control adaptive immunity, we can design safe and effective vaccines composed of protein subunits.

LOCAL PLACEMENT OF B-CELL MEMORY

Long-term protection through immune memory requires an accelerated capacity for the response to rechallenge with the original, priming antigen. Our recent studies indicate that memory B cells develop in local draining lymphoid organs and that a significant cohort of memory B cells remains locally in these sites without recirculating. Upon antigen rechallenge, the response of local memory B cells is more vigorous and rapid if antigen reenters the system through the original portal of priming.

Hence, we have discovered a level of local organization that may underlie the rapidity of the responses of memory B cells and accelerate secondary immune responses. Appropriate placement of antigen-experienced memory cells may be a major attribute of immune memory that reflects initial priming conditions and underpins a highly efficient capacity for local immunosurveillance. Therefore, priming location and placement of memory B cells become important considerations in priming regimens of protein vaccination.

REGULATING B-CELL FUNCTION

A specialized class of helper T cells called follicular B helper T (T_{FH}) cells regulate B-cell immunity. We recently described the local development of antigen-specific T_{FH} cells in response to vaccination with proteins. We found that the helper T cells with higher binding of peptide–MHC class II molecules to the T-cell receptors developed into effector T_{FH} cells in draining lymphoid tissues. The high-binding T_{FH} cells were retained in these sites for the induction of effector B-cell responses and B-cell memory. Importantly, our results indicate the existence of a memory counterpart for effector T_{FH} cells that are retained for extended periods in draining lymphoid tissues. These local memory T_{FH} cells had decreased effector capacity but rapidly regained functional attributes of effector T_{FH} cells when rechallenged with antigen.

The results of these studies and those of the research on B-cell memory described earlier suggest a new level of local organization for antigen-specific B-cell memory and the memory T_{FH} regulators of the immune response to vaccine boosters. Thus, the development and appropriate deployment of antigen-specific memory cells can substantially affect local immunosurveillance and the quality of long-term immune protection.

DENDRITIC CELLS AS ANTIGEN-SPECIFIC REGULATORS

The maturation of antigen-experienced dendritic cells is the central initiating event for adaptive immunity. We are examining the role of immune adjuvants, antigen dose, and route of immunization in shaping maturation of dendritic cells in vivo. The subsequent effect of these changes on selection of T-cell receptors and the function of antigen-specific T helper cells is an important ongoing focus of our research. Changes in the initial exposure to antigen also substantially affect the quality and quantity of effector B-cell responses and the development of memory B cells.

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Evolution of HIV-Host Interactions

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HIV type 1 (HIV-1), the cause of the AIDS pandemic, entered the human population in the last 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 that encodes the virus spike. The spike mediates 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 sensitivity of the virus to inhibitors that prevent cell entry and to neutralizing antibodies.

To more fully understand the cost of each mutation associated with changing coreceptor binding from CCR5 to CXCR4, we have reconstructed all possible mutational pathways between 4 parental CCR5-using viruses and their CXCR4-using descendants separated by 3–7 mutations. We used site-directed mutagenesis to introduce all possible combinations of single and multiple mutations in the HIV-1 envelope gene. The mutated envelopes were combined with an envelope-deficient reporter virus to make HIV-1 particles capable of only a single cycle of infection.

Mutations in variable loops 1 and 2 of the envelope protein improved the use of CCR5 but did not permit infection via CXCR4. Mutations in variable loop 3 led to use of CXCR4 for virus entry, but only very poorly. Combinations of mutations in all 3 variable loops improved the ability of the virus to use CXCR4. The sequence in which mutations were introduced was critical. Thus, the probability of coreceptor switching is constrained by having to make the right mutation at the right place at the right time.

We also mapped the domains of CCR5 and CXCR4 required for infection by each of the viruses with mutated envelopes by using chimeric coreceptors with the 4 extracellular domains derived from either CCR5 or CXCR4. The initial stage of coreceptor switching favored use of the second extracellular domain of CXCR4, and 2 mutants required only this domain for infection.

These results emphasize the major fitness costs of envelope evolution and the constraints that may explain the many years that transpire between primary infection and emergence of CXCR4-using viruses.

We did an extensive functional analysis of the mutated envelopes to examine other costs of viral evolution. An expected finding was that apparent CCR5 binding affinity was diminished before CXCR4 binding was gained, with the consequence that infection mediated by viruses with mutated envelopes became particulary sensitive to CCR5 inhibitors. Unexpected findings were that CD4 binding affinity increased during the transition from use of CCR5 to use of CXCR4, perhaps to compensate for poor coreceptor affinity. Moreover, sensitivity to neutralizing antibodies also increased, suggesting that antibodies could be a selective force against coreceptor switching. We also analyzed use of coreceptors other than CCR5 or CXCR4 during coreceptor switching and found preserved use of CCR3 that increased before switching and was maintained after CXCR4 use improved.

These studies were initially performed with HIV-1 envelopes from subtype B; up to 50% of patients with this subtype eventually have evolution of virus to use CXCR4. We recently extended these studies to subtype C viruses, for which use of CXCR4 is much less frequent. Use of alternative coreceptors appears to be common in the small subset of subtype C envelopes that can mediate infection via CXCR4. These observations suggest that the ability of a virus to use multiple alternative coreceptors other than CCR5 may provide an evolutionary advantage.

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Control of Cytokine Expression by Arginine Methylation and Citrullination

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Helper T cells can be divided into 2 distinct populations on the basis of their immune specificity and cytokine profiles. Type 1 helper T cells produce IFN-γ and are responsible for cell-mediated immunity; type 2 helper T cells secrete IL-4 and are associated with the humoral immune response. These 2 types of cells have been associated with susceptibility to malignant, infectious, allergic, and autoimmune diseases.

The improper development of type 2 helper T cells can lead to allergy and asthma, and an overactive response by type 1 helper T cells can lead to autoimmune diseases such as type 1 diabetes.

Because of the opposing roles of the 2 types in immune function, the development and migration of helper T cells must be tightly regulated. Indeed, the discrete subsets, type 1 and type 2, reciprocally antagonize the maturation and behavior of each other in the immune response, resulting in a population of helper T cells that is primarily type 1 or type 2. Thus, manipulating the ratio of type 1 to type 2 helper T cells provides an intriguing avenue of therapy, and understanding the molecular events that control lineage-specific cytokine expression may provide useful tools to modulate the helper T cell response.

Although several lineage-specific and nonspecific transcription factors are required for the development and function of type 1 and type 2 helper T cells, less is known about the events that occur after the reactivation of type 1 and type 2 effector populations and result in the disparate cytokine profiles of the 2 types of helper T cells. Signal transduction pathways use posttranslational modifications to translate changes in the extracellular milieu into environment-sensitive gene expression in a timely and efficient fashion.

Phosphorylation of serine, threonine, and tyrosine residues and protein ubiquitination have been widely studied. We are interested in 2 different modifications of arginine residues: methylation and citrullination. Arginine methylation of proteins by members of the protein arginine methyltransferase (PRMT) family regulates subcellular localization of the methylated proteins and modulates protein-protein interactions. Less is known about the function of citrullination by members of the peptidyl arginine deiminase (PAD) family, but clearly PAD activity can antagonize arginine methylation.

We discovered a unique contribution of arginine methylation and citrullination to cytokine gene expression downstream of signaling by T-cell receptors. Our goal is to investigate more broadly the role for arginine methylation and citrullination in immune function, including further study of helper T cells and other immune cell types. We are determining upstream regulation of PRMT or PAD expression and activity and are characterizing the effects of ablation or suppression of PRMT or PAD expression. Understanding the role of posttranslational modifications, such as arginine methylation and citrullination, of proteins that are key in regulating cytokine production will give us novel targets in diseases induced or exacerbated by the cytokine environment, such as inflammatory arthritis.

Analysis of Immune Learning in B Lymphocytes

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

The development of B lymphocytes is a multistep process punctuated by the somatic generation of genes for antibody heavy and light chains 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 carrying 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 chain 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 recent 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 "proofreading" 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 NF-κB and rel transcription factors may be involved in both positive and negative regulation of the *RAG* genes. We have also made progress in our understanding of 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 Cγ2 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 produced mice with a defect in this editing. These mutant mice lack a functional recombining sequence/κ light-chain–deleting element, which is involved in destructive editing of loci for κ light chains in cells that go on to rearrange either a second allele for κ light chains or genes for λ light chains. These mice are being assessed for their ability to produce autoantibodies and to accelerate autoimmune disease when crossbred with mice prone to autoimmunity.

In other studies, we 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 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, in the past year we 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 B. Beutler and coworkers, Department of Immunology. The findings suggest that additional adjuvant signaling pathways may await discovery. Perhaps such putative pathways could be exploited to provide novel ways to boost the efficacy of vaccines.

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Unique Mechanisms of Virus Neutralization by the Adaptive and Innate Immune Systems

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Control of virus infection by high-affinity neutralizing antibodies often occurs via intervention in the earliest steps in cell entry, such as receptor blockade or membrane penetration. In contrast, we found that a neutralizing monoclonal antibody directed to the major outer capsid protein (hexon) of adenovirus does not interfere with attachment of virus to cell receptors, internalization of virus, or escape of virions from endosomal compartments. Instead, the antibody blocks a relatively late step in cell entry involving transport of partially disassembled adenovirus particles to the nucleus mediated by dynein motors and microtubules. This entry step is also used by diverse viral pathogens for entry and/or cell egress. In biochemical studies, we showed that the neutralizing antibody substantially increased virus association with microtubules and micro-

tubule-associated proteins. Further studies are in progress to pinpoint the defect in cytoplasmic transport of virus particles. Our results reveal an unanticipated mode of antibody-mediated virus neutralization.

Specific elements of the innate immune system also contribute to virus inactivation, although the mechanisms involved are not fully elucidated. We recently showed that human α-defensin 5, a small, naturally occurring antimicrobial peptide, causes dose-dependent inhibition of adenovirus infection. This peptide inhibits multiple types of adenovirus, including types 5 and 35. In mechanistic studies, we showed that the defensin does not restrict virus attachment or internalization but instead efficiently blocks escape of virus from the early endosome. Human α-defensin 5 stabilizes the virus particle, as indicated by resistance to temperature-mediated capsid disassembly. Increased capsid stability impedes the release of the membrane lytic protein VI, thereby blocking escape of virions from endosomes. Further studies are in progress to solve the structure of human α-defensin 5 in complex with adenovirus. The knowledge gained from these studies not only will help define the role of the innate immune response in viral infections but also may lead to the development of novel antiviral compounds that restrict late events in virus entry.

Biochemical and Genetic Analyses of Adenovirus-Mediated Membrane Penetration

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Compared with the accumulated knowledge on membrane fusion by enveloped viruses, relatively little information is available on the mechanisms by which nonenveloped viruses penetrate cell membranes. We showed that protein VI, a capsid protein located in the interior of the adenovirus capsid, plays a key role in disruption of host cell membranes. During cell entry, the adenovirus vertex region undergoes disassembly, allowing release of protein VI from the interior of the virus concomitant with disruption of the endosome. In recent studies, we found that mutations introduced into the putative lipid-binding domain of protein VI reduce the membrane lytic activity of the protein.

Studies are in progress to introduce these mutations directly into the protein VI–coding region in adenovirus DNA to create mutant viruses with decreased infectivity and endosome-disrupting activity. In further investigations, we will use x-ray diffraction techniques to study the structure of wild-type and mutant forms of protein VI. Finally, protein VI has been incorporated into cell-derived nanoparticles (vaults) to develop novel cargo devices for gene transfer. These studies should increase our knowledge of how nonenveloped viruses penetrate cell membranes and may facilitate the development of nonviral methods of gene transfer.

Structure Analyses of Wild-type and Mutant Adenoviruses

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We recently determined the structure of a human adenoviral vector at 6.9-Å resolution, one of the largest macromolecular complexes analyzed by using electron cryomicroscopy. These studies allowed us to refine the location of multiple adenovirus capsid proteins, including protein VI, a molecule that plays a role in membrane disruption during cell entry. The results also suggested that protein IIIa acts as a linchpin for the assembly of the virus vertex region, which includes penton base, fiber, proteins IIIa and VIII, and the peripentonal hexons.

To gain a better understanding of adenovirus assembly and disassembly, we have also used electron cryomicroscopy to analyze the structure of a temperature-sensitive mutant adenovirus designated *ts*1. This virus is hyperstable and contains multiple unprocessed precursor capsid proteins. Consequently, *ts*1 particles do not undergo disassembly in the endosome and lack the ability to escape this compartment during infection. In preliminary structure analyses, the *ts*1 particle had a much stronger and well-defined density in the core region than the wild-type virus did. Our working hypothesis is that one or more of the unprocessed precursor capsid proteins links the core region with the outer surface of the virion, thereby resulting in a stronger reconstruction of the core. Further analyses at 10-Å resolution are planned to gain further insights into adenovirus assembly and disassembly.

To complement the electron cryomicroscopy structural studies, we have produced crystals of intact virus

particles that diffract to approximately 5-Å resolution. We have also generated electron density maps of adenovirus particles at 7.5-Å resolution that reveal striking features of the virion core, including the putative location and arrangement of the viral chromosome. Further studies are in progress to improve resolution to reveal the precise location and orientation of the viral capsid proteins and the adenovirus DNA within the core.

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Characterization of Stem Cell–Like Cancer Cells in Immunocompetent Mice

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The cancer stem cell hypothesis has recently gained considerable recognition as the descriptor of tumorigenesis. According to this hypothesis, tumor initiation and growth are driven by a small population of stem cell–like cancer cells (SCLCCs) in the tumor and the specific phenotypes of these cells are responsible for the escape of tumor cells from conventional therapies, resulting in disease relapse.

To develop novel approaches to target SCLCCs in an effort to suppress breast cancer metastasis in mice, we characterized these stem cells in immunocompetent mice. We were able to identify and purify SCLCCs from 4T1 murine breast carcinoma cells, in the absence of expression of surface markers, by using the phenomenon that stem cells generally can efflux lipophilic fluorescent dyes such as HOECHST 33342.

We found that efflux of this dye correlated with the expression of ABCG2, a member of a family of membrane transport proteins that use the energy of ATP hydrolysis to transport various molecules across the membrane, and could be inhibited with calcium channel blockers. This HOECHST^{low} population of cells was designated the side population and was greatly enriched for stem cells in the hematopoietic system within the bone marrow. We found that the dye-based side population technique could be used in flow cytometry analysis and cell sorting to isolate SCLCCs from the 4T1 breast carcinoma cells that could be transplanted into the immunocompetent microenvironment of syngeneic BALB/c mice. Indeed, flow cytometry analysis and cell sorting clearly defined a side population that constituted 0.15% of the cells within normal mouse bone marrow and 2.21% of cells within 4T1 cells.

The side population phenomenon was verified by the finding that coincubation of side population cells with the calcium channel blocker verapamil significantly reduced the number of cells by more than 90%. Furthermore, the side population of cells from 4T1 cells had a Sca-1^{high}e–Kit^{-}CD45^{-} phenotype, characteristic for SCLCCs, and reverse transcription–polymerase chain reaction also revealed upregulation of ABCG2, Sca-1, Wnt-1, and transforming growth factor β in the side population cells but not in other cells. Additionally, 4T1 side population cells revealed increased resistance to chemotherapeutic agents and had an increased ability to efflux doxorubicin, findings that correlated with a selective increase in the percentage of side population cells found in the tumors of doxorubicin-treated mice. Most importantly, side population cells, but not other cells, had a markedly higher repopulation and tumorigenic potential in vivo, characteristics that correlated with an increased number of cells in the side population compartment of tumors derived from side population cells.

Together, these results indicate that we have successfully characterized SCLCCs from the spontaneously metastasizing 4T1 breast carcinoma cell line in the immunocompetent microenvironment of syngeneic mice. The finding of upregulated expression of Wnt-1 by 4T1 subpopulation cells provides the rationale for future research, namely to establish proof of concept that specific inhibition of Wnt-1 signaling in SCLCCs leads to the induction of apoptosis and suppression of metastases of 4T1 breast carcinoma cells.

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Protective Signaling by the Protein C Pathway in Endothelial Cells

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Current evidence indicates that cellular signaling by activated protein C (APC), an anticoagulant serine protease, has a protective role in disorders in which the vascular barrier separating the blood from tissues is compromised, such as sepsis or ischemia-reperfusion injury in the brain. So far, APC is the only drug approved for treatment of 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), a member of the G protein–coupled 7 transmembrane domain receptor family. This signaling pathway has been implicated in protective effects of APC infusion in mouse models of stroke and sepsis.

Our concept is that the thrombin receptor PAR-1 evolved to sense the balance of thrombin and APC activity in the endothelial microenvironment. 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 pro-apoptotic 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 recently showed 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, such 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. Interestingly, recent results indicate that APC-cleaved PAR-1 accumulates on the cell surface even in the presence of thrombin (Fig. 1). The molecular mechanisms leading to distinct receptor trafficking of thrombin- vs APC-cleaved PAR-1 remain to be elucidated. 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 pro-

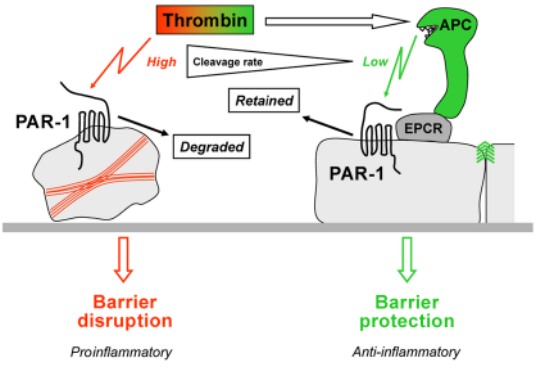

Fig. 1. Inflammatory disorders such as sepsis are associated with increased permeability of the endothelial cell monolayer at the blood-tissue interface. APC enhances endothelial barrier integrity dependent on binding to EPCR and activation of PAR-1. Thrombin–PAR-1 signaling leads to barrier disruption. 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 is 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.

tective 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 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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The Scripps Research Institute Molecular Screening Center

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The Scripps Research Institute Molecular Screening Center is a national center for small-molecule screening and is part of the National Institutes of Health (NIH) Molecular Libraries Screening Centers Network of the NIH Roadmap. The Scripps center is distributed between the California and the Florida campuses; its component parts are assay implementation, chemistry, ultra-high-throughput screening, and pharmacokinetics. These 4 cores are unified by the informatics core in Florida, which provides a single data environment.

The mission of the Scripps center is to use the NIH library of more than 100,000 individual compounds to screen molecular and cell-based targets, which are accepted through an NIH-wide peer-reviewed application process, for proof-of-concept small-molecule probes. The Scripps center is a leading, full-production NIH center, and researchers at the center have successfully identified and published information on proof-of-concept molecules. Last year, the center completed 21 full-deck screens for 8 different types of molecular targets. Compounds discovered by this process are public information that can be accessed by all scientists through the PUBCHEM database of the National Center for Biotechnology Information.

The Scripps center now integrates expertise in small-molecule discovery and optimization with state-of-the-art robotics and informatics. The center is well poised to provide new insights into the basic science of small-molecule probes of physiologic and pathologic function that can move scientific fields forward, and we hope, over time, provide new, significant insights into therapies for human diseases.

Proof-of-Concept Chemical and Genetic Approaches to Signaling Lipids in Health and Disease

H. Rosen, G. Sanna, E. Jo, P. Gonzalez-Cabrera, A. Don, S. Cahalan, D. Marsolais, S. Brown, M-.T. Schaeffer, J. Chapman

IMMUNOSUPPRESSION AND VASCULAR INTEGRITY

Lymphocytes develop in the thymus (T cells) and bone marrow (B cells) and upon maturation egress from their sites of development to enter the bloodstream. Because the numbers of lymphocytes with specific receptors for antigen are limited, the probability of random productive collision of specific lymphocyte, antigen, and antigen-presenting cell in a permissive environment for an efficient immune response is low. In the immune system, this probability is enhanced by rapid recirculation of lymphocytes through secondary lymphoid organs, so that each lymphocyte has many opportunities to respond to its specific antigen. A sufficient number of blood lymphocytes are therefore essential for the development of efficient immune responses and are maintained by the recirculation of lymphocytes through the secondary lymphoid organs.

Using small synthetic druglike organic molecules, we elucidated specific molecular gatekeepers that control the number of recirculating lymphocytes. These compounds alter lymphocyte trafficking and induce clinically useful immunosuppression by activating a single sphingosine 1-phosphate (S1P) receptor subtype, $S1P_1$. Using 2-photon fluorescence and selective agonists and antagonists of this receptor, we directly imaged the control of lymphocyte egress from lymph nodes in living systems.

MOLECULAR CONTROL OF LYMPHOCYTE MIGRATION

Molecular control of the migration of subsets of lymphocytes within the recirculation pathway is a fundamental issue of therapeutic importance. Although transplantation involves the sensitization of an immuno-

logically naive host, treatment of most autoimmune diseases requires intervention in a sensitized host that already has autoreactive effector T cells in the periphery. We approached this problem by examining the role of the S1P system in the control of lymphocyte egress from lymph nodes and thymus, and using chemical approaches, we revealed differences between intrinsic lymphocyte and barrier mechanisms that alter lymphocyte migration. The rapid reversibility of agonist-mediated lymphocyte arrest coupled with its competitive reversal by molar excess of antagonist in vivo strongly supports an endothelial barrier mechanism.

ROLE OF SIGNALING LIPIDS IN CONTROL OF VASCULAR INTEGRITY

Modulating vascular barriers is of high therapeutic interest. Enhancement of the integrity of capillary barriers protects against important inflammatory diseases of the lung, such as acute respiratory distress syndrome, and modulation or opening of the blood-brain barrier may be useful in enhancing drug entry into the brain. We have used biosynthetic enzymes and agonists, antagonists, and genetic deletions of receptors to study the role of the signaling lipid S1P system.

$S1P_1$, found on lung capillaries, tightens capillary junctions and protects from leakage. Antagonists of $S1P_1$ promote lung leakage from the vascular side and have an essential role in tonic activation of receptors by signaling ligand in blood to maintain capillary integrity. The S1P-receptor axis and its level of tonic activation may therefore be an important independent variable in the control of capillary barrier function, and its activation and modulation in serious human diseases such as acute respiratory distress syndrome are under study.

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Proteases in Thrombosis, Inflammation, and Cancer

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REGULATION OF TISSUE FACTOR–INITIATED THROMBOSIS BY PROTEIN DISULFIDE ISOMERASE

The key event in the initiation of coagulation is formation of the ternary complex of tissue factor (TF) with enzyme coagulation factor VIIa and substrate factor X. We identified a novel regulatory mechanism for TF by showing that protein disulfide isomerase (PDI) switches TF from coagulation to signaling by targeting the allosteric disulfide Cys^{186}-Cys^{209} in the TF extracellular domain.

We further characterized the interaction of purified PDI with TF and found that PDI, independent of its disulfide/thiol isomerase activity, has an activating effect on the coagulant activity of TF. Mutation of specific residues in TF abolished activation by PDI, providing evidence that PDI primarily interacts with TF, rather than with enzyme or substrate in the ternary TF initiation complex. PDI had no effect on fully active TF, confirming the original data that PDI predominantly associates with cryptic, inactive TF. PDI enhanced TF coagulant activity on microvesicles that are shed from cells and circulate in the blood. These experiments indicate that PDI plays an important role as an activating chaperone for cryptic blood-borne TF.

PROTEASE SIGNALING IN ANGIOGENESIS AND CANCER

Coagulation and other proteases are ubiquitously generated during wound repair, neovascularization, and cancer invasion. Proteases cleave and activate the G protein–coupled protease-activated receptors (PARs). We studied the roles of PAR-1 and PAR-2 in ocular angiogenesis and in subcutaneous tumor growth models.

Deletion of the cytoplasmic domain of TF produces enhanced PAR-2–dependent angiogenesis, providing a sensitized model of PAR-2 signaling in pathologic neovascularization. In hypoxia-driven angiogenesis of oxygen-induced retinopathy, revascularization of areas of central vaso-obliteration occurred significantly faster in mice lacking the cytoplasmic domain of TF than in

wild-type animals. Deletion of PAR-2, pharmacologic inhibition of the TF-VIIa complex, and blockade of tyrosine kinase receptor pathways with imatinib, an antitumor drug, reversed accelerated angiogenesis in the mutant mice. These data indicate that TF-VIIa activates PAR-2 to promote angiogenesis in the context of growth factor signaling.

Although the thrombin receptor PAR-1 has been implicated in signaling endothelial cell progenitors of relevance for angiogenesis, deletion of PAR-1 produced no delay in ocular angiogenesis or tumor growth, an angiogenesis-dependent process. Thus, direct TF signaling through PAR-2, rather than thrombin signaling, can be the predominant pathway in angiogenesis.

TF SIGNALING AS A REGULATOR OF SEVERE SYSTEMIC INFLAMMATION

In severe systemic inflammation associated with viral hemorrhagic fevers and sepsis, TF expression leads to protracted activation of intravascular coagulation that contributes to escalation of inflammation. To study the interplay of coagulation activation and inflammation, we established a highly reproducible model of severe systemic inflammation and coagulation activation. Using this model, we found that compared with wild-type mice, mice lacking the cytoplasmic domain of TF had enhanced and prolonged systemic activation of coagulation. This phenotype allowed us to determine whether increased activation of coagulation leads to amplification of inflammation. We found no effect of increased intravascular coagulation on the onset or severity of early systemic inflammation, indicating that the coagulation cascade does not directly amplify the direct effects of lipopolysaccharide signaling. Surprisingly, in this model, inflammation resolved earlier in the mutant mice than in wild-type mice, and the mutant animals were protected from death due to sepsis. These data indicate that escalation of inflammation is regulated by the TF pathway.

Attenuation of coagulation activation with specific inhibitors had no effect on recovery from the severe inflammatory injury, implicating TF-dependent signaling. Consistently, in this sepsis model, inhibition of direct TF signaling abolished the protective phenotype of mice lacking the cytoplasmic domain of TF. These experiments revealed an unexpected regulatory role of direct TF signaling in the exacerbation of severe systemic inflammation. We will use this established model to further evaluate the basic mechanisms by which TF and PAR signaling regulate inflammation and to test therapeutic approaches.

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Structural Analysis of the Host-Pathogen Interface

E. Ollmann Saphire, D.M. Abelson, J.M. Dias, M.L. Fusco, J.E. Lee, C.R. Kimberlin, K.M. Weinell

We 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 against the viruses as the microbes exist naturally 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

Ebola virus has reemerged, with outbreaks causing 50%–90% mortality. Survival depends on the ability of the host to mount early and strong immune responses. However, the viral nucleocapsid proteins VP35 and VP24 block innate immune signaling. 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.

An additional feature of Ebola virus, which may confound immune recognition, is that the viral glycoprotein occurs in 2 different structural forms. The minor form, transmembrane GP, functions in attachment and entry and would be the relevant target for antibody neutralization. However, the major, secreted form, sGP, is the predominant antibody target. We are developing a large panel of antibodies against Ebola virus to determine which epitopes are unique to the viral surface GP and are recognized by rare, neutralizing antibodies. Structures of GP and sGP, alone and in complex with these antibodies (Fig. 1), should explain how 2 struc-

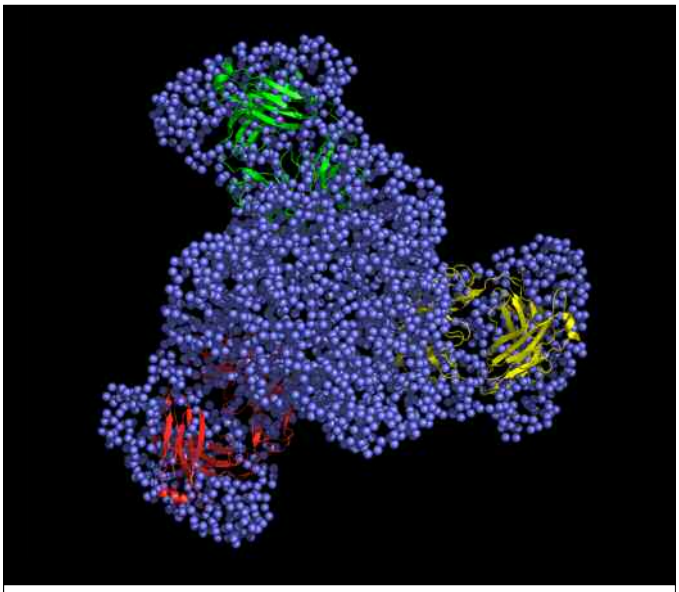

Fig. 1. Model, generated by small-angle x-ray scattering, of the trimeric Ebola virus glycoprotein in complex with 3 neutralizing Fab fragments.

tures arise from the same sequence, provide templates for the design of vaccines, and illustrate structural mechanisms by which the virus escapes immune surveillance.

DENGUE VIRUS

Dengue virus is a mosquito-borne flavivirus that causes up to 100 million infections, resulting in either dengue fever or dengue hemorrhagic fever, each year. The more severe dengue hemorrhagic fever is linked to secondary infection and is associated with cross-reactive antibodies. To aid in vaccine design, we are determining crystal structures of envelope proteins of contemporary field isolates of dengue virus, alone and in complex with antibodies, to characterize structural features of epitopes associated with neutralization and enhancement.

LASSA VIRUS

Lassa virus causes as estimated 150,000 infections per year in West Africa, with a 10%–15% mortality. We are determining crystal structures of the Lassa virus glycoprotein spike alone and in complex with neutralizing antibodies and the Old World arenavirus receptor α-dystroglycan. Structural information will aid in the development of effective treatments and will provide insight into effective strategies for vaccine design.

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Mechanisms of Antigen Receptor Signaling in T-Cell Development and Function

K. Sauer, Y.H. Huang, Y. Yang

T cells defend against infections by recognizing pathogen-derived antigens through the T-cell receptor (TCR). Perturbed TCR signaling can result in immunodeficiency or in the emergence of T cells that recognize innocuous antigens as foreign, resulting in allergies or autoimmune diseases. Our core interests are the mechanisms by which TCR signaling directs T-cell development and function. By identifying novel genes involved in TCR signaling and analyzing the function of the encoded proteins, we hope to improve understanding of the molecular mechanisms that regulate T-cell development, function, and malfunction in disease.

To identify such genes and develop hypotheses about their functions, we take advantage of collaborative functional genomics approaches and complement these with detailed, hypothesis-driven mechanistic analyses of selected genes. For example, detection of mutant mice deficient in inositol 1,4,5-trisphosphate 3-kinase B (ItpkB) in a forward genetic screen 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, TCR-induced membrane recruitment of the protein tyrosine kinase Itk through the PH domain of the kinase is perturbed in ItpkB$^{-/-}$ 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 the function of PIP_3-binding PH domains likely is a global regulatory mechanism.

Detailed follow-up showed that this novel IP_4 function is essential for positive selection, a process whereby functional double-positive cells are induced to mature into functional T cells. Positive selection is mediated by TCR activation of the Ras–extracellular signal–regulated kinase pathway. In double-positive cells, ItpkB

establishes a feedback loop of phospholipase C γ1 (PLCγ1) activation through Itk that is essential for production of the second messenger DAG, a critical Ras activator (Fig. 1).

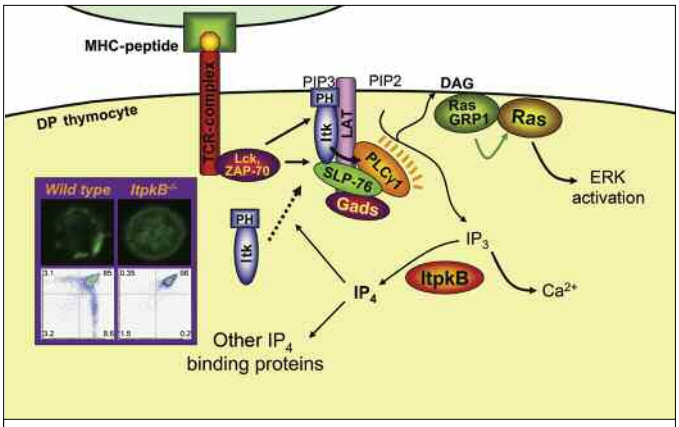

Fig. 1. Proposed role for ItpkB in a positive feedback loop sustaining TCR-induced DAG production in double-positive (DP) thymocytes undergoing positive selection. TCR stimulation results in the activation of proximal tyrosine kinases (Lck, ZAP-70); phosphorylation of transmembrane adaptor proteins, including LAT; assembly of signaling complexes centered at LAT; and low-level activation of Itk and its effector PLCγ1. Low PLCγ1 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 or TCR contact-site translocation, LAT/PLCγ1 signaling complex interactions, and activation of Itk. This allows Itk to fully activate PLCγ1, 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 immunofluorescence microscopy, coincides with a block of T-cell development at the $CD4^+CD8^+$ double-positive stage, shown via flow cytometric analysis.

Phenotypic differences between mice lacking the gene for Itk and those lacking the gene for ItpkB suggest that IP_4 mediates positive selection through additional targets besides Itk. We are now identifying these targets and defining the precise molecular mechanisms through which IP_4 regulates recruitment of PH domains and positive selection in thymocytes.

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Consequences of T-Cell Recognition of Self-Antigens and Tumor Antigens

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The 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. If a $CD8^+$ T cell recognizes antigen on a quiescent dendritic cell with relatively low levels of expression of costimulatory molecules, then activation of the T cell results in deletion and tolerance. Inflammatory signals, such as those due to the presence of pathogens or activated lymphocytes or genetic alterations that lead to chronic inflammation, activate dendritic cells to express cell-surface costimulatory molecules and cytokines. If $CD8^+$ T cells recognize antigen on activated dendritic cells, the costimulatory molecules and cytokines prevent deletion and promote the clonal expansion of the T cells and the development of effector functions.

Understanding the signals that result in either T-cell deletion or immunity is of importance 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 transgenic self-antigen (influenza virus hemagglutinin) expressed by the insulin-producing beta cells in the pancreatic islets in 3 different types of mice: normal mice, diabetes-prone nonobese diabetic mice, and mice in which the beta cells express an oncogene that promotes spontaneous transformation and production of tumors.

In all 3 types of mice, the interaction between antigen and naive $CD8^+$ T lymphocytes specific for hemagglutinin first occurs in the pancreatic lymph nodes. There, antigen is recognized on dendritic cells that obtain it from beta cells in the islets and cross-present

it to naive T cells in the lymph nodes. In normal mice, this interaction results in an abortive activation of the T cells and subsequent deletion of the potentially autoreactive T cells specific for hemagglutinin.

MECHANISMS OF PROTECTION FROM TYPE 1 DIABETES BY GENETIC POLYMORPHISMS

The spontaneous diabetes that develops in nonobese diabetic 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 humans and in 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, 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 nonobese diabetic 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 nonlymphoid bone marrow–derived cells, possibly the antigen cross-presenting dendritic cells.

ROLE OF $CD4^+$ HELPER T CELLS IN PROMOTING TUMOR CELL DESTRUCTION BY $CD8^+$ T CELLS

$CD4^+$ helper T cells can enhance the performance of $CD8^+$ T cells in different ways, including enhanced clonal expansion during activation of $CD8^+$ T cells, enhanced tissue infiltration by activated effector $CD8^+$ T cells, and enhanced survival of the effector cells. We are assessing the ability of $CD4^+$ helper T cells at various times after activation of $CD8^+$ T cells to evaluate the ability of the helper cells to promote destruction of tumor cells by $CD8^+$ T cells. One way $CD4^+$ T cells help tumor-specific $CD8^+$ T cells is by facilitating entry of the $CD8^+$ cells into the tumor tissue. This process is much less efficient if no $CD4^+$ helper T cells are present within the tumor environment.

CROSS-PRESENTATION OF N-TERMINAL ELONGATED NOMINAL PEPTIDE

In cross-priming, antigen-presenting dendritic cells acquire, process, and present antigens scavenged from other cells and use these antigens to activate naive $CD8^+$ T cells. Previous studies have indicated that nominal peptide is inefficiently cross-presented and that proteins and large polypeptides that require proteosomal processing are the main source of naturally cross-presented antigens. N-terminal extension of nominal peptide by as few as 3 residues is sufficient to produce a substrate for transporter associated with antigen processing–dependent cross-presentation that is highly efficient in cross-priming murine $CD8^+$ T cells in vivo. On a molar basis, cross-priming with trimer-extended peptide is 20-fold more efficient than priming with intact protein. This method of peptide extension should be useful in facilitating in vivo studies of $CD8^+$ T-cell immunity and tolerance that rely on cross-presentation.

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Regulation of Homeostasis of Mature T Cells

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Homeostasis of mature T cells is largely governed by 2 related cytokines, IL-7 and IL-15, which bind to receptors belonging to the common γ-chain (CD132) receptor family. Other members of the CD132 family include receptors for IL-2, IL-4, IL-9, and IL-21. In essence, survival of naive T cells, which persist in interphase, is controlled by signals from contact with IL-7 and self-peptide–MHC ligands. Memory $CD8^+$ T cells, which are at a higher state of activation than naive T cells are, depend on both IL-7 and IL-15, but not MHC ligands, for survival and for their periodic basal homeostatic proliferation. Under normal conditions, both IL-7 and IL-15 are thought to be produced at a constitutive level by non–T cells, namely epithelial, stromal, and antigen-presenting cells, and the established basal level supports survival of a

finite numbers of T cells. Consistent with the idea that T cells continually use up these cytokines to stay alive, the levels of the cytokines in an individual increase with depletion of T cells. The increased cytokine availability in turn induces the remaining T cells to undergo spontaneous acute homeostatic proliferation to restore the T-cell numbers to near-normal levels.

Among various subsets of T cells, memory $CD4^+$ T cells are probably the least understood in terms of their homeostatic requirements. IL-7 plays a major role; memory $CD4^+$ T cells express high levels of the receptor for IL-7 (CD127), and lack of IL-7 abbreviates the life span of the cells. In contrast, IL-15 probably does not play a role, because memory $CD4^+$ T cells express only low levels of the receptor for IL-15 (CD122); instead, signals from contact with MHC molecules are thought to be involved. One problem with these conclusions is that they were based on studies with memory-phenotype $CD4^+$ T cells that arise spontaneously in the absence of any intentional immunization.

Notably, when antigen-specific bone fide memory $CD4^+$ T cells were studied, it became apparent that these cells require both IL-7 and IL-15, but not MHC molecules, for their homeostasis. Thus, in the absence of either IL-7 or IL-15, antigen-specific $CD4^+$ T cells have a short life span and undergo severely diminished homeostatic proliferation. In the absence of both cytokines, the life span is further curtailed, and homeostatic proliferation ceases completely, even in the presence of MHC molecules. Because IL-15 supports survival of many cell types, especially memory CD8+ T cells, natural killer T cells, and natural killer cells, that express much higher levels of CD122 than memory $CD4^+$ T cells do, memory $CD4^+$ T cells are at a distinct disadvantage in their ability to compete for IL-15 under normal conditions. This situation could explain why the longevity of memory $CD4^+$ T cells is generally less stable than that of memory $CD8^+$ T cells. The interesting issue of how many subsets of lymphocytes persist by requiring a similar set of homeostatic factors, IL-7 and IL-15, without blatantly affecting survival of each other is currently unknown.

Despite the increasing understanding of the importance of homeostatic cytokines for T-cell biology, the clinical use of the cytokines is yet to be realized, and, at least for IL-2, is fraught with severe side effects. A major problem is that these cytokines are probably produced and presented to T cells in a particular manner under in vivo conditions, and cytokines injected systemically do not behave in the same way. One approach to overcome this difficulty is to inject the cytokine after it is bound to a monoclonal antibody specific for the cytokine.

For several cytokines, including IL-2, IL-4, and IL-7, we found that cytokine-antibody complexes had 100-fold more biological activity when injected than the injected free cytokines did. For IL-15, similar increased activity can be achieved by binding to its natural presenting molecule, the IL-15 receptor α-chain. These cytokine-antibody or cytokine-receptor complexes can be used to strongly enhance T-cell responses.

Interestingly, IL-2, which can be recognized by either the trimeric or the dimeric IL-2 receptors, can either enhance or suppress immune responses, depending on the specificity of the monoclonal antibody to IL-2. This difference occurs because one type of IL-2–antibody complex preferentially engages the dimeric receptor on memory $CD8^+$ cells, and the other type binds the trimeric receptor on regulatory T cells. Thus, the immune system can be either enhanced (e.g., for treatment of cancer) or suppressed (e.g., to treat autoimmune diseases), depending on the particular IL-2–antibody complexes administered. The clinical usefulness of these and other cytokine-antibody complexes are currently being explored.

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Structure-Function Studies of Innate and Adaptive Immunity

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ACTIVATION OF T-CELL RECEPTORS

Our goal is to understand the molecular switches that lead to activation of T cells. We have achieved assembly of functional complexes of T-cell receptors (TCRs) on artificial bilayers with recombinant forms of TCRαβ, CD3ζε, CD3γε, and CD8αβ. We use single-molecule, multicolor imaging by 2-photon and total internal reflection fluorescence microscopy, in collaboration with K. Fish, University of Pittsburgh, Pittsburgh, Pennsylvania, and electron microscopy to examine the interactions 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 be the elementary switches required for T-cell activation. This hypothesis is supported by our 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 CD8αβ.

AUTOIMMUNE DIABETES

We are using MHC multimers to detect antigen-specific T-cell populations in diabetes-prone nonobese diabetic mice. 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 depleting 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 T cells. The specificity of the intervention will limit side effects and complications of general immunosuppression. Structure determination of complexes containing TCRs and diabetogenic MHC molecules (Fig. 1) is under way and will help in understanding the link between the genes for MHC class II molecules and autoimmunity.

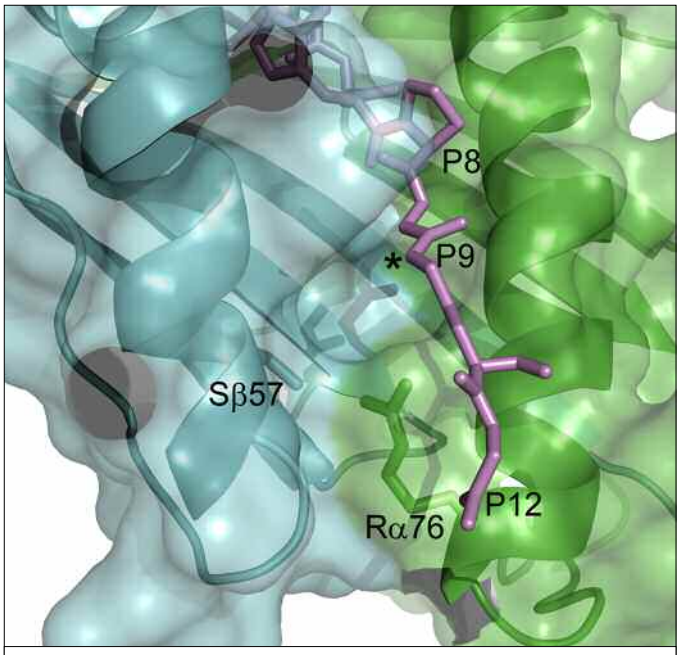

Fig. 1. Top view of the P9 region of the diabetogenic I-A^{g7} molecule. Unlike all other MHC class II molecules, diabetogenic MHC class II molecules lack a negatively charged residue at position β57 (serine substitution), the outer boundary of the P9 region.

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 α-galactosylceramide and isoglobotrihexosylceramide (β-linked) by a TCR bearing a unique α-chain (Vα14 in mice, Vα24 in humans) and a limited set of Vβ 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 and P. Tobias, Department of Immunology, 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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Genetics of Systemic Autoimmunity and the Role of Cytokines and T-Cell Homeostasis in Autoimmunity and Cancer

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We have continued our research on systemic autoimmunity, such as systemic lupus erythematosus (SLE), by addressing the genetic basis, the role of Toll-like receptors (TLRs) and interferons, homeostatic T-cell disturbances, defects in B-cell tolerance, and the effects of blocking cyclin kinase inhibitors or transmethylation reactions. We have also investigated provoking beneficial autoimmunity for tumor therapy.

GENETICS OF SYSTEMIC AUTOIMMUNITY

We are defining the role and identity of susceptibility genes that promote both spontaneous and induced SLE in mouse models. Previously, we identified lupus-related loci in 4 major lupus-prone strains of mice (NZB, NZW, BXSB, and MRL) and C57BL/6 mice, which are not predisposed to autoimmune disease, and a disease-resistance locus in DBA/2 mice. We are characterizing several of these loci in interval congenic lines, including the NZB/NZW-related *Lbw2*, *Lbw5*, and *Lbw7* (chromosomes 4, 7, and 1, respectively), the MRL/B6-*Fas^{lpr}*–related *Lmb1–Lmb4* (chromosomes 4, 5, 7, and 10), and the NZB-related *Hmr1* (chromosome 1). We are also analyzing smaller interval substrains to more precisely map SLE traits.

We cloned *Lmb3* and discovered that it is a spontaneous nonsense mutation of the gene for coronin-1A, an actin-binding protein, in the less lupus-prone C57BL/6-*Fas^{lpr}*/Scr substrain, indicating that *Lmb3* is a disease-modifying or resistance allele. The mutation leads to the accumulation of cellular F-actin in lymphoid cells and 3 intrinsic defects in T cells: impaired migration, increased apoptosis, and reduced antigen receptor–mediated activation associated with a defect in peak calcium flux. These defects result in reduced numbers of T cells, impaired T cell–dependent humoral responses, and ultimately suppression of autoimmu-

nity. *Lmb3* is one of the few SLE-associated loci identified by genome-wide screening for which the genetic basis has been determined and confirmed. The finding of a disease-inhibiting mutation is also of interest, because such mutations are clinically important in infectious diseases but have rarely been identified in autoimmunity. Finally, our results provide a strong rationale for investigating the role of actin-regulatory proteins in autoimmunity and the potential of these proteins as therapeutic targets.

TLR-DEPENDENT AND TLR-INDEPENDENT INDUCTION OF TYPE I INTERFERON AND ITS RELEVANCE TO SYSTEMIC AUTOIMMUNITY

We and others have shown that type I interferons (IFN-α/β) play central roles in the pathogenesis of SLE in predisposed mouse strains and humans. To gain insight into the etiology of SLE and other autoimmune diseases and to elucidate pathways that might be targeted for therapy, we are investigating what induces the production of IFN-α/β.

We have used lupus-prone mice (C57BL/6-*Fas*^{lpr}) congenic for the *3d* mutation to define the role of endogenous nucleic acids and TLRs in SLE. This research is a collaboration with B. Beutler and associates, Department of Immunology, who used *N*-ethyl-*N*-nitrosourea mutagenesis to identify a mutation of the gene *Unc93b1* that causes absence of signaling through endosomal TLR3, TLR7, and TLR9 and decreased presentation of exogenous antigens by MHC class I and class II molecules.

Indeed, the *3d* congenic mice had significant decreases in the levels of autoantibodies, proteinuria, and mortality. The reduced disease manifestations seemed to be due to defective TLR signaling rather than to inefficient MHC class II presentation, because *3d* and wild-type mice had similar antibody responses when immunized with a thymus-dependent antigen. The maintenance of normal humoral responses despite the compromised MHC presentation likely was due to compensatory changes in the T-cell repertoire, because *3d* mutant CD4$^+$ T cells had higher proliferative responses than did wild-type CD4$^+$ cells upon antigen presentation by antigen-presenting cells from wild-type or mutant animals. Additionally, chronic injections of lipid A, an agonist that acts through TLR4, which is not compromised by the *3d* mutation, did not induce sustained increases in IgG antibodies to chromatin in *3d* mutant C57BL/6-*Fas*^{lpr} mice but did in wild-type controls. These findings clearly indicate that signaling through endogenous nucleic acids recognizing endosomal TLRs is required for full expression of lupus-like systemic autoimmunity. In further research in collaboration with E. Janssen, La Jolla Institute for Allergy and Immunology, La Jolla, California, and Dr. Beutler and associates, we found that apoptotic cell debris could induce production of IFN-α/β by an immature type of lymphoid dendritic cells and that this response was TLR-independent.

On the basis of these findings and other findings in the literature, we formulated a 2-phase model to explain the mechanism by which apoptotic materials and associated nucleic acids induce IFN-α/β and precipitate SLE (Fig. 1). The initial TLR-independent phase is mediated by uptake of apoptotic cell debris and associated nucleic acids by dendritic cells, whereas the subsequent TLR-dependent phase has an amplification function and is mediated by uptake of TLR ligands derived from self-antigens (principally nucleic acids) complexed with autoantibodies. This model suggests that antagonists for TLRs or interferons may be appropriate for the treatment of SLE and other autoimmune diseases. Alternatively, removing or reducing sensing of endogenous macromolecular structures that initiate production of IFN-α/β might be a novel therapeutic approach

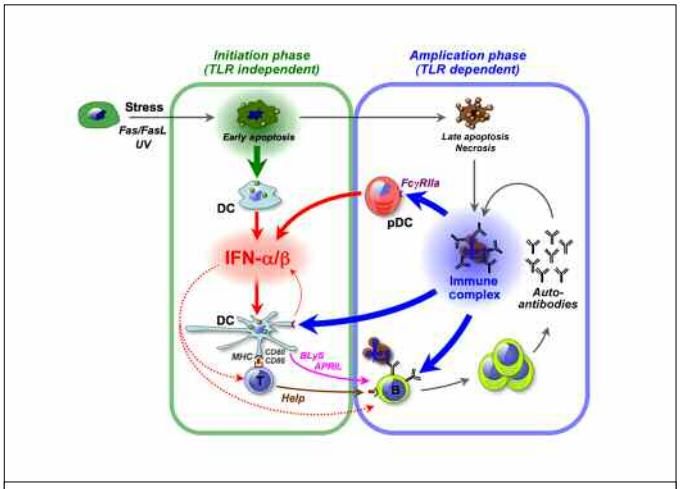

Fig. 1. Model of the 2-phase induction of IFN-α/β in systemic autoimmunity. The initiation phase is TLR independent and mediated by apoptotic cell material taken up by a specialized subset of lymphoid dendritic cells (DCs), leading to production of IFN-α/β. Under the effect of IFN-α/β, lymphoid and myeloid dendritic cells upregulate MHC and costimulatory molecules and differentiate into efficient self-antigen–presenting cells, leading to activation of quiescent autoreactive helper T cells. As a result of T helper cells, effects of BLys and APRIL, and dual engagement of B-cell receptors and TLRs, autoreactive B cells proliferate and differentiate into plasma cells. After the formation of immune complexes containing nucleic acids, the amplification phase is induced, which encompasses TLR-dependent induction of IFN-α/β in plasmacytoid (pDC) and conventional dendritic cells, and enhanced B-cell proliferation and autoantibody production.

that has the advantage of not compromising the otherwise beneficial function of TLR and interferons.

T-CELL HOMEOSTASIS AND SLE

Memory T cells accumulate slowly over the normal human life span and become the dominant phenotype only at an advanced age. In contrast, in systemic autoimmunity, the accumulation of T cells with a memory-like phenotype is highly expedited. This accumulation, thought to be promoted by diverse and ever-present self-antigens, has been attributed to several pathogenetic mechanisms that act singly or in combination.

Currently, we are examining whether this disturbance of T-cell homeostasis could also be a consequence of abnormalities in levels of trophic cytokines, IL-7 and IL-15, and/or expression of the cytokine receptors. We found that the phenotype of the accumulating T cells in the MRL-*Fas*^{lpr} strain of lupus-prone mice is compatible with activation by homeostatic proliferation rather than classical antigen-driven stimulation. Although peripheral CD4$^+$ and CD8$^+$ T cells of these mice expressed the receptors for IL-7 and IL-15, the massively expanded CD4$^-$CD8$^-$ double-negative T cells were devoid of these receptors, suggesting that survival of double-negative T cells does not depend on IL-7 or IL-15. The absence of these receptors on double-negative T cells may lead to excesses in T-cell trophic cytokines and promote survival and proliferation of autoreactive single-positive T cells, thereby sustaining the autoimmune process. Accordingly, in vivo inhibition of IL-7 signaling via antibodies to the cytokine's receptor reduced lymphoproliferation and other autoimmune manifestations in MRL-*Fas*^{lpr} mice. Therefore, blockade of receptors for cytokines that sustain T-cell survival and proliferation may have marked mitigating effects in autoimmune lymphoproliferative syndromes in humans.

TOLERANCE OF IGG-SWITCHED AUTOREACTIVE B CELLS AND DEFECTS IN LUPUS-PRONE MICE

Foreign antigens trigger the growth and differentiation of antigen-specific memory B cells in peripheral lymphoid organs. On rechallenge with the original antigen, memory B cells produce high-affinity isotype-switched antibodies with novel effector functions and prime the immune system for rapid and effective responses. Although useful, somatic mutation may lead to acquisition of self-reactivity, raising the question of how this potentially harmful event is controlled. To address this issue, in collaboration with D. Nemazee, and M. McHeyzer-Williams, Department of Immunology, we produced transgenic mice that express a membrane-tethered γ2a-reactive superantigen (γ2a-macroself-antigen) and assessed the fate of IgG2a-expressing cells induced by immunization with a T cell–dependent antigen. We found that self-reactive IgG2a-switched B cells were deleted, leading to the absence of memory responses for this subclass. Overexpression of *Bcl2* in B cells partly overcame this regulation. Tolerance mediated by this macroself antigen was inefficient in the MRL-*Fas*^{lpr} lupus-prone mice, suggesting that defective deletion of isotype-switched autoreactive B cells is central to Fas mutation–associated systemic autoimmunity.

CYCLIN KINASE INHIBITORS AND AUTOIMMUNITY

We previously showed that a deficiency in the cell-cycle inhibitor p21 in lupus-prone BXSB male mice is associated with enhanced apoptosis of T and B lymphocytes, decreased numbers of activated/memory CD4$^+$ T cells, and reduced autoimmunity. These findings were consistent with our hypothesis that repeated stimulation by self-antigens of CD4$^+$ T cells in SLE leads to a buildup of cell cyclin kinase inhibitors, such as p21 and p27, and to the accumulation of cells in a replicative senescence-like state. Such cells would be resistant to apoptosis and proliferation but upon stimulation could produce autoimmunity-promoting proinflammatory factors. We have now completed studies of BXSB mice deficient in the cell cyclin inhibitor p27. We found that unlike a deficiency in p21, the lack of p27 had no effect on disease progression or severity. Moreover, a modest enhancement of disease occurred in p21-deficient type 1 diabetes–prone nonobese diabetic-prone mice. These findings suggest that the role of p21 is contextual, depending on the underlying genetic defect or defects that drive the autoimmune process.

INDUCTION OF ANTITUMOR AUTOIMMUNITY

We previously showed that vaccination with tumor cells coupled with lymphopenia-induced homeostatic proliferation can produce effective antitumor autoimmune responses in mice. We hypothesized that this beneficial effect is due to a reduction in the activation threshold of low-affinity T cells that recognize self-tumor antigens. We used a breast carcinoma model in mice to examine the applicability of this approach for treatment of metastasizing tumors. Although the size of the primary tumor was reduced, metastasis was only marginally affected, probably because of the time required for recovery in the number of lymphocytes and acquisition of a diverse repertoire. To potentially overcome this problem, we adapted a protocol based on the findings of our collaborators, C.D. Surh, Department of Immunology; O. Boyman, University of Lausanne, Lau-

sanne, Switzerland; and J. Sprent, Garvan Institute, Sydney, Australia; that complexes of IL-2 and/or IL-7 with corresponding antibodies induce extensive proliferation of $CD8^+$ T cells in nonlymphopenic hosts. We found that the expanded T cells had characteristics of activated and effector T cells, and treatment with such complexes (particularly complexes consisting of IL-2 and antibody to IL-2) reduced the size of the primary tumor, lung metastasis, and mortality. These findings provide clinically relevant approaches for breaking tolerance to tumor antigens in humans with malignant tumors.

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Molecular Mechanisms of Host-Pathogen Interactions

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Infection by microbial pathogens often sets in motion chains of events that cause severe injury to the host, and nowhere is this phenomenon illustrated more dramatically than in the response by humans to infection by gram-negative bacteria. In his book *Lives of a Cell*, Lewis Thomas characterizes the host response to the endotoxin, or lipopolysaccharide, of gram-negative bacteria as being "read by our tissues as the very worst of bad news. . . . There is nothing intrinsically poisonous about endotoxin, but it must look awful, or feel awful, when sensed by cells. Cells believe that it signifies the presence of gram-negative bacteria, and they will stop at nothing to avoid this threat." In other words, the innate immune response to infection has caused a serious disease in humans.

Clearly, much human suffering could be eased if such overzealous host responses could be tempered. However, such responses, when not overzealous, are a normal part of the host's homeostatic mechanisms, designed to respond to the threat of infection by gram-negative bacteria. Accordingly, we are attempting to define the mechanisms of innate immunity and learn how to control innate immune responses without compromising host defenses against pathogens. Recently, we contributed to the understanding of innate immunity through studies of Toll-like receptors (TLRs) and effector mechanisms that mediate host responses to infection.

It is now well appreciated that the innate immune system is positioned at the intersection of multiple host pathways, including those for microbial and viral recognition, enhancement of adaptive immune responses, and, possibly, cancer immunosurveillance. Each pathway depends on ligand recognition by specific cellular receptors that are either membrane bound (plasma membrane as well as endosomal compartments) or cytosolic. The TLRs are the most important class of membrane-bound receptors. Among cytosolic receptors, an important family known as the NLR/Nod/Caterpillar family has been identified. Within this family, 2 proteins, Nod1 and Nod2, are involved in recognition of bacterial ligands distinct from the ligands for TLRs. Activation of TLR and Nod signaling pathways leads to production of multiple cytokines with proinflammatory and anti-inflammatory activities. Such responses are central to host responses to infection. However, when a breakdown occurs in the normal regulatory mechanisms that control these pathways, disease may result.

Perhaps the most well-understood link between innate immunity and human disease is in the host response to infection. When dysregulation of innate immune responses occurs, clinical syndromes such as septic shock and acute respiratory distress syndrome ensue. Dysregulation of innate immune responses may also play a role in human diseases in which chronic inflammation is responsible for disease progression, including autoimmune and autoinflammatory diseases. Genetic studies in humans have revealed strong associations among various members of the Nod family of proteins and human diseases.

During the past year, we made considerable progress in several different areas. We have identified several unique pathways of innate immunity that link plant and

mammalian innate immunity pathways. Our research on the pathways in mammals include studies of the COP9 complex and the role of SGT1 in Nod1 signaling. These findings are documented in our recent publications.

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Targeting Tumor-Associated Macrophages

R. Xiang, Y. Luo, H. Zhou, J.A. Krueger, C.D. Kaplan

Immunization against molecules prominently expressed by tumor-associated macrophages (TAMs) is a novel antitumor strategy; it leads to a remodeling of the tumor microenvironment that attracts TAMs and mediates their function. TAMs, which consist primarily of polarized M2 ($F4/80^+/CD206^+$) macrophages, have unique characteristics that make them excellent targets for cancer immunotherapy. First, they are only marginally cytotoxic for tumor cells because of the limited production of nitric oxide and proinflammatory cytokines by TAMs. Second, these macrophages have poor antigen-presenting capability but strongly promote tumor growth and metastasis because they effectively suppress T-cell activation by secreting many growth and proangiogenic factors. Third, TAMs are involved in signaling pathways that regulate the functions of fibroblasts in the tumor stroma.

We hypothesized that a reduction in the density of TAMs in the tumor stroma decreases the release of factors that potentiate tumor growth and angiogenesis. This reduction, in turn, should cause remodeling of the tumor microenvironment and markedly suppress tumor cell proliferation, vascularization, and metastasis. Testing this hypothesis was facilitated by our discovery that legumain, an asparaginyl endopeptidase and a member of the C13 cysteine protease family, is specifically overexpressed on TAMs, where it functions as a stress protein and as a superb target for cancer immunotherapy. We found that an oral legumain-based DNA vaccine induced a strong $CD8^+$ T cell–mediated immune response against TAMs. This response dramatically reduced the density of TAMs in tumor tissues and resulted in a marked decrease in the levels of proangiogenic factors released by these macrophages, such as transforming growth factor β, TNF-α, matrix metalloproteinase 9, and vascular endothelial growth factor. This decrease, in turn, led to a marked suppression in both tumor angiogenesis and tumor growth and metastasis. Importantly, we showed the success of this strategy in murine models of metastatic breast, colon, and non–small cell lung cancers; 75% of vaccinated mice survived lethal tumor cell challenges, and 62% were completely free of metastases.

In conclusion, decreasing the number of TAMs in the tumor stroma effectively altered the tumor microenvironment involved in tumor angiogenesis and progression to markedly suppress tumor growth and metastasis. We anticipate that gaining further insights into the mechanisms required for an effective antitumor strategy may ultimately lead to novel therapeutic targets and more effective anticancer strategies.

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Neutralizing Antibodies Against HIV Type 1 gp41

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To date, candidate vaccines against HIV type 1 (HIV-1) typically have not protected against disease and have elicited relatively weak neutralizing antibody responses. We and others think that a better understanding of HIV-1 neutralization will facilitate the development of more effective vaccines. We have focused on the transmembrane glycoprotein gp41, which together with the surface glycoprotein gp120 forms a heterotrimer (gp41-gp120)3 on the surface of the virion; this trimer is the exclusive target of HIV-1 neutralizing antibodies.

The glycoprotein gp41 is relatively conserved, making it an attractive vaccine target. In particular, well-described monoclonal antibodies, 2F5 and 4E10, which bind within the highly conserved membrane-proximal external region (MPER) of gp41, can neutralize a wide range of primary isolates of HIV-1. Unfortunately, simply immunizing animals with recombinant gp41 or unconstrained MPER peptides does not elicit significant neutralizing antibody responses, most likely because of poor mimicry of the native conformation of gp41. Although the native structure of gp41 remains unknown, structure-function analyses of the neutralizing monoclonal antibodies are helping to unravel the elusive structural details of the MPER.

We identified a human monoclonal antibody, Z13, that also binds to the MPER. Using in vitro mutagenesis and affinity selection, we recently identified a variant, Z13e1, that binds with 100-fold higher affinity to gp41 than parental Z13 does and can neutralize primary isolates of HIV-1. However, the neutralization potency of Z13e1 is still several-fold lower than that of 2F5 or 4E10. Using a combination of peptide mapping, mutagenesis, monoclonal antibody competition, and neutralization assays, we have identified the minimal epitope of Z13e1, which lies between those of 2F5 and 4E10. These data have led us to conclude that the epitope of Z13e1 is partially occluded on the native envelope trimer. We are now collaborating with I.A. Wilson, Department of Molecular Biology, to obtain a crystal structure of Z13e1 in complex with an MPER peptide to provide us with the structural details of the gp41-binding site of Z13e1.

We have also identified monoclonal antibodies against a receptor-activated form of gp41 within the N-heptad repeat region. These antibodies have modest activity against HIV-1 and will be useful in elucidating the structure and conformational changes of gp41 that lead to fusion. We intend to expand our efforts to identify monoclonal antibodies against the N-heptad repeat region and MPER. The results of a thorough structure-function analysis of many such monoclonal antibodies will then be applied to the design and evaluation of more precise structural mimics of native and receptor-activated epitopes on HIV-1 gp41. We envision that such a monoclonal antibody "feedback" approach will lead to better vaccine candidates.

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