OBJECTIVES AND SCOPE

The great advances in immunology in recent years have made this field one of the most rapidly growing in the biological sciences. This remarkable growth has been stimulated by the influx of investigators from other disciplines such as biochemistry, genetics, molecular biology, and by an increased number of investigators who came to immunology through the more traditional routes of microbiology and various medical disciplines. As a consequence, immunology has become a vast and rich field encompassing outlooks that range from the highly clinical to the highly molecular. Although such perspectives appear to be too diverse, they are, in fact, highly interdependent. The understanding in molecular terms of the cellular events of the immune response will be critical for the elucidation of the molecular mechanisms of immunological disorders. For these reasons, Critical Reviews in Immunology will seek to present a balanced overview of contemporary immunology and blend together molecular immunology and immunobiology.

The journal is published bimonthly and is one volume, which is divided into six issues, per year. The topics and their authors are proposed by the members of the Editorial Board of distinguished and active immunologists.

Review articles — The journal publishes critical and timely review articles in various aspects of contemporary immunology. These review articles constitute the major part of any given issue. It is hoped that the articles will provide a critical analysis rather than a passive account of information in a given topic. The articles are rigorously refereed by outstanding and expert investigators. Review articles are, therefore, by invitation and unsolicited papers cannot be considered. However, proposals for reviews are encouraged and will be seriously considered by the Editorial Board. A proposal should give a short description (about one page) of the intended work.

The phenomenal advances in the field of immunology make it a dynamic and continuously changing field. With such an explosion of knowledge and information, concepts and dogmas often lag behind and might even become outdated. To allow constructive discussion among immunologists and to maximize exchange of ideas the journal will publish (in addition to the review articles) the following:

Opinions/hypotheses — These are proposed concepts, based on sound experimental data that should be available in the scientific literature. The hypothesis, which does not necessarily have to be based on the author’s own experimental work, should outline consequences that could be tested by experiments. Opinions and hypotheses will be subject to the usual peer review process.

Letters to the editor — These could be responses to hypotheses, clarifications, viewpoints, and other such matters that will encourage discussion. This is an open forum. The letters, however, should be scholarly, and personal attacks or abusive style will not be permitted. Letters to the editor will be peer reviewed.

News and comments — These will include important news items such as major breakthroughs and new discoveries in immunology and even other biological sciences that might impact on the field of immunology. Summaries of national and international meetings are also appropriate news items.

Book reviews and books received — Authors and publishers of immunology books are invited to submit their books for review and evaluation by the journal. Usually, one or two reviews can be assigned to a given issue. However, as a service to the scientific community, all books that are received will be listed.

Calendar of events — As a service to the scientific community, the journal will list titles and dates of immunology meetings up to one year in advance. Organizers of future meetings are encouraged to submit title, date, location, and name/address/telephone number of the person to contact for further information. There will be no charge for this service.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Article Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Role of CD8 T-Cell-Mediated Autoimmune Diseases of the Central Nervous System</td>
<td>311</td>
</tr>
<tr>
<td>Martina Deckert, Monica Sanchez-Ruiz, Anna Brunn, &amp; Dirk Schlüter</td>
<td></td>
</tr>
<tr>
<td>Genetic Control of Dβ Reading Frame and Its Effect on B-Cell Development and</td>
<td>327</td>
</tr>
<tr>
<td>Antigen-Specific Antibody Production</td>
<td></td>
</tr>
<tr>
<td>Harry W. Schroeder, Jr., Michael Zemlin, Mohamed Khass, Huan H. Nguyen,</td>
<td></td>
</tr>
<tr>
<td>&amp; Robert L. Schelonka</td>
<td></td>
</tr>
<tr>
<td>Tumor Antigen Presentation by Dendritic Cells</td>
<td>345</td>
</tr>
<tr>
<td>Troels R. Petersen, Nina Dickgreber, &amp; Ian F. Hermans</td>
<td></td>
</tr>
<tr>
<td>The Role of Chemokines in Migration of Metastatic-like Lymphangioleiomyomatosis</td>
<td>387</td>
</tr>
<tr>
<td>Cells</td>
<td></td>
</tr>
<tr>
<td>Gustavo Pacheco-Rodriguez and Joel Moss</td>
<td></td>
</tr>
</tbody>
</table>
Role of CD8 T-Cell-Mediated Autoimmune Diseases of the Central Nervous System

Martina Deckert1*, Monica Sanchez-Ruiz1, Anna Brunn1, & Dirk Schlüter2
1 Abteilung für Neuropathologie, Universitätsklinikum Köln, Köln, Germany; 2 Institut für Mikrobiologie, Otto-von-Guericke Universität Magdeburg, Magdeburg, Germany

*Address all correspondence to Martina Deckert, MD, Abteilung für Neuropathologie, Universitätsklinikum Köln, Kerpener Str. 62, D-50924 Köln, Germany; Tel.: 49-221-478-5265; Fax: 49-221-478-7237; Email: neuropatho@uni-koeln.de.

ABSTRACT: In T-cell-mediated autoimmune diseases of the central nervous system (CNS), CD4 T cells have long been regarded as the only pathogenetically relevant T-cell population. However, growing clinical and experimental evidence suggests that CD8 T cells also contribute significantly to autoimmune responses in the CNS. We discuss the potential induction of autoimmune CD8 T cells by infections, the impact of the microenvironment of the CNS on CD8 T-cell responses, and the potential interaction of CD8 T cells with autoantigen-expressing resident brain-cell populations—neurons in particular—in light of clinical and experimental findings.

KEY WORDS: CNS, autoimmunity, CD8 T cells, neuron, astrocyte, infection, Listeria monocytogenes, molecular mimicry

I. INTRODUCTION

T-cell-mediated autoimmune diseases include a broad spectrum of disorders and may affect a variety of organs, including the heart, joints, pancreas, and bowel, as well as the central nervous system (CNS). For many human diseases, excellent animal models that have allowed insight into the principles of the underlying pathogenesis are available. There is strong evidence for a decisive role of CD4 T-helper 1 (Th1) and Th17 CD4 T cells, which in the context of major histocompatibility complex (MHC) class II antigens mediate, sustain, and coordinate autoimmune inflammation.1 Multiple sclerosis (MS) is the most intensely studied disorder of the CNS. The crucial role of CD4 T cells in MS has been confirmed by studies in rat and mouse models of experimental autoimmune encephalomyelitis (EAE), which can be induced either by adoptive transfer of CD4 T cells specific for myelin protein(s) or by active immunization with peptides of CNS myelin proteins. Although CD8 T cells outnumber CD4 T cells in MS lesions, their contribution to immunopathology has been regarded as merely regulatory, if relevant at all. However, there is now growing evidence for a pathogenetically important role of CD8 T cells in MS. Current knowledge indicates a dual role for CD8 T cells in autoimmune diseases of the CNS: suppressive and stimulatory. This review focuses on CD8 T cells in cerebral autoimmune responses. After a general discussion of the poten-
tial mechanisms of how autoreactive CD8 T cells may be induced and how CNS-specific features (including immunoprivilege) may affect autoreactive T-cell responses, the pathogenetic role of CD8 T cells in various clinical and experimental autoimmune CNS disorders is discussed.

A. Infections and Autoimmune Diseases

Clinically, the association between infections and autoimmune disorders is a well-known phenomenon. Viral, bacterial, and parasitic pathogens may cause autoimmune inflammation of a variety of organs, including the heart and bowel, as well as the peripheral and CNS. Furthermore, epidemiological studies have revealed that, clinically, MS and diabetes mellitus may be triggered by previous viral infections; for example, it has been suggested that there is an association between MS and Epstein-Barr virus (EBV) infection. A connection between an infection and a monophasic episode of autoimmune demyelination is illustrated by acute disseminated encephalomyelitis, a postinfectious encephalitis characterized by multiple foci of perivenous demyelination, which characteristically follows a viral infection and/or immunization. Acute disseminated encephalomyelitis is frequently, but not exclusively, associated with measles infection. Another clinically important autoimmune disease of the peripheral nervous system is Guillain-Barré syndrome, a flaccid muscle paralysis that may be triggered by a gastrointestinal infection with Campylobacter jejuni.

With the exception of the aforementioned disorders, intense efforts to identify a (single) underlying pathogen have failed in the vast majority of autoimmune disorders. This may be due to a long latency period between the initial infection, during which time the causative agent has successfully been eliminated, and clinical manifestation of the autoimmune disease. In addition, the initial infection may have been clinically asymptomatic, which makes it more difficult to identify the causative agent.

Two major hypotheses have been raised to explain how infections may induce autoimmunity in the CNS and other organs: bystander activation and molecular mimicry. Although infectious diseases may trigger cerebral autoimmune diseases by other mechanisms, such as activation of intracerebral antigen-presenting cells or inhibition of immunosuppressive cytokines, bystander activation and molecular mimicry are currently the most intriguing and most likely mechanisms.

1. Bystander Activation of Autoreactive T cells and Epitope Spreading

It is well known that cytotoxic T cells are polyclonally stimulated during viral infections. Cytokines secreted by antigen-responsive cells at infectious foci may directly stimulate surrounding T cells by cytokines in the absence of direct triggering of the T-cell receptor. Thus, a proinflammatory microenvironment creates a fertile field, allowing activation of autoreactive, but not pathogen-specific T cells, which subsequently may damage host cells. This latter scenario may become particularly relevant when underlying infection causes tissue destruction, thereby deliberating host cell proteins, which can be presented by antigen-presenting cells to autoreactive bystander T cells. However, in general, bystander activation of T cells is not of major biological importance, as was demonstrated in a transgenic model of lymphocytic choriomeningitis virus (LCMV)-induced diabetes mellitus.

With respect to the CNS, a critical role for bystander activation of T cells underlying human autoimmune disorders has not yet been identified. However, in the CNS of mice, bystander damage has recently been demonstrated in Borna virus infection. After viral clearance from the brain, bystander neurons in the CA1 sector of the hippocampus, which did not express Borna virus antigen, were specifically attacked by CD8 T cells and underwent apoptosis. In this scenario, collateral damage of neurons was attributed to a T-cell-mediated impairment of astrocytic function, which resulted in a microenvironment that negatively affected survival of CA1 neurons. However, it has been shown that the overall contribution of bystander damage in the CNS may be rather small.

In addition, autoreactive T cells may be induced by epitope spreading. During infection, pathogen-specific T cells develop in a hierarchical
manner, being directed against immunodominant epitopes first. Subsequently, the T-cell response may be generated against further, less dominant epitopes of the same protein or against epitopes of a different protein. Such epitope spreading is useful for the host to optimize a T-cell response during an ongoing infection, but bears the unwanted risk of stimulation of potentially harmful autoreactive T cells. Epitope spreading, combined with an increased amount of host cell epitopes generated by antigen-presenting cells from destroyed host cell tissue and the adjuvant effect on an infection, may create a fertile field for the development of cerebral autoimmune reactions. The concept of epitope spreading has been confirmed experimentally in EAE and Theiler’s murine encephalomyelitis virus (TMEV)-induced demyelinating disease. In the latter model, a myelin-specific CD4 T-cell response evolved secondarily during CNS infection. Interestingly, in TMEV infection, epitope spreading preferentially targets myelin epitopes to which little T-cell tolerance is induced. So far, however, epitope spreading has only been demonstrated for CD4 T cells, not for CD8 T cells, in cerebral autoimmune diseases.

2. Molecular Mimicry

The attractive hypothesis of the concept of molecular mimicry is based on the existence of structural similarities between antigenic determinants of a pathogen and the host. Consequently, a single T-cell receptor may bind to structurally related antigens, which may differ in their amino acid sequence, bound to one or several MHC molecules. This T-cell-receptor degeneracy implies that T-cell responses to pathogen-specific antigens may result in the activation and expansion of T cells cross-reactive with self-antigens. T-cell-receptor recognition is remarkably flexible: a single T-cell receptor is able to respond to different peptides and can react with different peptide-MHC complexes of similar charge distribution and overall shape. The concept linking autoimmunity to molecular mimicry and T-cell receptor degeneracy has been substantiated in the work of Oldstone and Fujinami.

Experimentally, the concept of molecular mimicry has been proven for CD4 T cells in autoimmune demyelination of the CNS. TMEV expressing a proteolipid mimic (PLP139-151) induces CD4 T-cell-mediated autoimmune demyelination in the CNS. In addition to CD4 T cells, T-cell-receptor degeneracy has also been shown for CD8 T cells. Sandalova et al. demonstrated peptide-molecular mimicry-induced CD8 T-cell cross-reactivity. Despite large sequence disparity, rDBM, a rat peptide homolog of mouse dopamine b-mono-oxygenase, and LCMV gp33 peptides were presented in a nearly identical manner by H-2Db. The T-cell receptor-interacting surfaces of H-2Db.rDBM and H-2Db.gp22 MHC complexes were very similar with regard to shape, topology, and charge distribution, thus providing a structural basis for CD8 T-cell activation by molecular mimicry and subsequent development of autoreactivity. Furthermore, Fiorillo et al. demonstrated that a pathogen (EBV)-derived peptide can exhibit MHC class subtype-dependent, distinct binding modes, and that cytotoxic T-lymphocyte cross-reactivity between a viral peptide (pLMP2) and a self-peptide (pVIPR) in the context of MHC class antigens (HLA-B27) are allele-dependent properties.

However, for cerebral autoimmune disorders, the concept of molecular mimicry for CD8 T cells is still a matter of debate. Interestingly, a dysregulated EBV infection has been hypothesized to play a role in MS immunopathology. In addition to claiming a role for B cells in MS, Serafini et al. observed activated CD8 T cells at an increased frequency in EBV-high MS cases. These investigators suggested CD8 T cell-mediated immunopathology as a major determinant of tissue destruction in EBV-associated diseases, and as a contributor to the damage to myelin sheaths and axons in MS. Nevertheless, the concept of molecular mimicry has not yet been confirmed experimentally for CD8 T cells. In fact, most models demonstrating that pathogen-specific CD8 T cells can induce autoimmune disorders are rather models of “molecular identity” than “molecular mimicry.”

Evans et al. demonstrated that CD8 T cells induced immunopathology in mice transgenically expressing a protein of LCMV under the MBP promoter in oligodendrocytes. Interestingly, peripheral (intraperitoneal) infection resulted in an increase of CD8 T cells and an up-regulation of MHC class I and II molecules in the brain. Only after reinfection with either the same or an unrelated virus was full demyelination induced.
In addition, we demonstrated that infection with ovalbumin-expressing *Listeria monocytogenes* induced CD8 T-cell autoimmune disease in mice expressing ovalbumin transgenically in neurons.\(^{18}\)

### B. Prerequisites for Autoimmune Reactions in the CNS

Discussing potential mechanisms for how infections might trigger T-cell-mediated autoimmune diseases of the CNS, it should be stressed that the CNS is an immunoprivileged organ characterized by down-regulation of the immunological phenotype under normal conditions.

### 1. Immune Privilege of the CNS

To initiate an autoimmune response in the CNS, T cells need to be reactivated in lymphatic organs outside of the CNS prior to homing to their target organ, where they must be reactivated by local antigen-presenting cells. The entry of T cells into the CNS is limited by the blood-brain barrier. Homing of T cells to the CNS has been precisely addressed in many studies previously.\(^{19}\) In addition to the identification of a variety of cell-adhesion molecules and a plethora of chemokines involved in binding to cerebral endothelial cells, which express complementary receptors, the fate of encephalitogenic T cells and their interactions with CNS structures has been tracked in detail using intravital two-photon imaging. Tissue-specific autoimmune disease was induced by CD4 MBP-specific T cells expressing green fluorescent protein.\(^{20}\) These cells initiated autoimmune lesions around pial veins after incoming T cells had systematically scanned vascular surfaces and the subarachnoid space. Thereafter, effector CD4 T cells established contact with antigen-presenting phagocytes on the abluminal vascular surface and the pial membrane, produced inflammatory mediators, and thereby stimulated the formation of inflammatory infiltrates.\(^{20}\) An additional study revealed that initial recruitment of autoreactive, CCR6-expressing Th17 cells is dependent on the secretion of CCL20 by plexus epithelial cells, resulting in the recruitment of autoreactive Th17 cells into the ventricular system. Subsequent recruitment of additional autoreactive T cells via parenchymal blood vessels into the CNS was CCR6/CCL20 independent\(^{21}\); however, these studies focused on CD4 T cells, particularly in the model of EAE, and thus it remains to be shown that these findings hold true for other diseases and CD8 T cells.

Another anatomical feature that contributes to the immunoprivileged status of the brain is its lack of a conventional lymphatic drainage, which limits the efflux of antigens from the CNS to lymphatic organs. However, elegant studies have demonstrated pathways of lymphatic drainage of antigen from the brain to lymphatics,\(^{22}\) including draining of CSF via the cribriform plate and nasal mucosa to cervical lymph nodes. Furthermore, interstitial fluid drains from the brain along capillary and artery walls, taking a perivascular route to cervical lymph nodes. While lymphocytes do not migrate from the brain to regional lymph nodes in any significant number, the drainage of soluble antigen appears to be sufficient to stimulate a T-cell-mediated reaction in the brain. Cervical lymph nodes may constitute a major source of lymphocytes targeting the brain, as has been shown in experiments using lymphadenectomy, which reduced the intracerebral autoimmune reaction in EAE with concomitant cryolesion by 50%. Thus, the CNS is not absolutely separated from the lymphatic system.

Immune privilege of the CNS is not exclusively based on the existence of the blood–brain barrier, as illustrated by the compartmentalization of immune privilege to specific anatomic structures of the brain.\(^{23}\) Immune privilege, which is not absolute, is confined to the CNS parenchyma and preserved under inflammatory conditions. Interestingly, within the CNS parenchyma, there is evidence for further compartmentalization with differences in the degree of leukocyte influx into the brain and spinal cord, as well as into the white matter and the grey matter, respectively.\(^{24,26}\) Establishment and maintenance of immune privilege results from a combined effort of resident cell populations of the CNS, in particular astrocytes and neurons, and is mediated via both soluble and cell-mediated interactions. In the normal murine brain, neurons express the potent immunosuppressive cytokines transforming growth factor-beta (TGF-β) and interleukin-10 (IL-10).\(^{27,29}\) Neuronal electrical activity suppresses the inducibility
of MHC class II antigens by microglia through electrical activity-related neurotrophin secretion by neurons, partly caused by agonism at the microglial p75 neurotrophin receptor. Conversely, neurons suppress microglial activation via a CD200-CD200L interaction. Furthermore, various neuropeptides and neurotransmitters are immunosuppressive, and prostaglandins, synthesized by both astrocytes and neurons, down-regulate microglial MHC class II expression and cytokine production.

2. Lack of Immunological Ignorance of the CNS

Despite its immunological privilege, the CNS is far from being ignored by the immune system. Instead, it is integrated into a complex network that serves to protect this highly vulnerable organ that is characterized by a limited regenerative potential. In the normal CNS, only low numbers of CD4 and CD8 T cells are present; most of them reside in the ventricular system or in the leptomeninges, while they are absent from the brain parenchyma. In the intact spinal cord, T cells reside mainly in the gray matter, with higher numbers of CD8 than CD4 T cells.

In addition, in exclusively extracerebral infections, the brain is surveilled by antigen-specific CD4 and CD8 T cells. In murine systemic listeriosis, Listeria-specific CD4 and CD8 T cells enter the brain and specifically home to those anatomic sites that represent the target structures for L. monocytogenes in cases of cerebral infection (i.e., the leptomeninges and the ventricular system with choroid plexus epithelium and ependyma) in order to protect these highly vulnerable structures of the brain (Fig. 1). This specific recruitment of antigen-specific T cells only to sites where cognate antigen is or may be expressed within the brain apparently illustrates a general phenomenon of antigen specificity governing (CD8) T-cell infiltration into the brain.

3. Resident Brain Cells As Target Cells of (Auto)Immune Reactions

In the CNS, several cell populations may potentially become a target for (auto)immune reactions. CD4 T cells require MHC class II antigen expression on their target cells, a criterion that is fulfilled by microglia in the CNS. With severe damage, a few astrocytes may additionally express MHC class II antigens. The range for potential intracerebral cell populations to be recognized by CD8 T cells is remarkably broader. Upon exposure to inflammatory stimuli, resident brain cells, including microglia, astrocytes, and oligodendrocytes, readily express MHC class I antigens and can now be recognized by CD8 T cells. Thus, these cell populations may be rendered sensitive toward a CD8 T-cell-mediated (auto)immune response, which may also be infection related.

While the participation of neurons in (auto)immune reactions was negated for a long time, evidence from both clinical observations and experimental studies has revolutionized this view. Neurons may well contribute in a highly specific manner to immune responses in the CNS, although their immunological reactions are more strictly regulated than other resident brain-cell populations. Under physiological conditions, neurons express neither MHC class I nor class II antigens. Therefore, they are a target cell for a plethora of pathogen, including viruses, bacteria such as L. monocytogenes, and parasites such as Toxoplasma gondii, and may serve as a safe harbor, enabling escape from elimination by the immune system. Viruses, including members of the herpes, paramyxovirus, rhabdovirus, picornavirus,
and arenavirus families, infect CNS neurons and are often capable of establishing latent or persistent infection in their target cells.\textsuperscript{44-46} Immunological control or even elimination of these pathogens is largely dependent on CD8 T cells, raising the possibility that not only irreversibly damaged neurons but also intact nerve cells are recognized by pathogen-specific CD8 T cells.

A central issue regarding CD8 T-cell-mediated autoimmune responses against neurons is whether neurons: i) provide a milieu allowing the functional activity of autoreactive T cells, ii) attract CD8 T cells by chemokines, and iii) express molecules including MHC class I antigen and Fas, which allow their direct interaction with CD8 T cells, and, subsequently, CD8 T-cell-mediated killing of neurons.

In response to infectious stimuli, neurons express cytokines and chemokines that support the intracerebral immune defense of the offending pathogen. For example, upon infection with measles and West Nile virus, neurons secrete CCL5 and CXCL10,\textsuperscript{47,48} which recruits effector CD8 T cells required for control of the infection.\textsuperscript{47} Thus, these data suggest that neurons may play a crucial and early role in the induction of immune responses to viral invasion, and that they contribute to their own defense.\textsuperscript{48} Remarkably, West Nile virus-infected neurons were shown to be the primary cellular source of CXCL10, and its expression was temporally correlated with virus entry and replication in the CNS\textsuperscript{49}; this induction was independent of adaptive immune responses.\textsuperscript{47} Interestingly, chemokine production of neurons is strongly determined by the underlying pathogen; for example, in contrast to West Nile virus infection, neurons do not produce significant amounts of chemokines in murine Toxoplasma encephalitis.\textsuperscript{50} In this chronic parasitic infection, astrocytes and microglial cells produce several cytokines in close association with parasites and inflammatory infiltrates.\textsuperscript{50}

The immunological plasticity of neurons is illustrated by the observation that cultivated cerebellar neurons, which constitutively expressed IL-1, IL-6, TGF-\(\beta\), macrophage inflammatory protein-1-alpha (MIP-1\(\alpha\)), and MIP-1\(\beta\), induced TGF-\(\beta\) and up-regulated MIP-1\(\alpha\) and MIP-1\(\beta\) expression upon infection with \textit{T. gondii}.\textsuperscript{43} In this model, the cytokine and chemokine pattern of neurons was further modified by interferon-gamma (IFN-\(\gamma\)) and tumor necrosis factor (TNF),\textsuperscript{43} two potent proinflammatory mediators that are produced by CD4 and CD8 T cells and macrophages/microglia during \textit{Toxoplasma} encephalitis and are indispensable for parasite control and survival.\textsuperscript{29,51,52}

In contrast to healthy neurons, irreversibly damaged neurons express fully functional MHC class I antigen, thus allowing their physical recognition by CD8 T cells.\textsuperscript{53} This observation is of fundamental importance for immune reactions to infectious pathogens with respect to both immune and autoimmune reactions. While normal neurons lack Fas expression, inflammatory stimuli and virus infection rapidly induce Fas on neurons,\textsuperscript{54-59} which allows their killing via the Fas/FasL apoptotic pathway by antigen-specific CD8 T cells.

The highly sophisticated interactions between neurons and CD8 T cells is also highlighted in herpes simplex virus (HSV) infection. After the lytic infection subsides, HSV persists in the neurons of spinal and cranial nerve sensory ganglia, together with both specific and nonspecific CD8 T-cell infiltrates.\textsuperscript{60} In latency during attempted viral reactivation, virus-infected neurons present antigen to CD8 T cells to keep them in a persistently activated effector memory state. CD8 T cells located in apposition to neurons secrete granzyme B, which under these conditions interferes with viral gene expression but does not induce apoptosis of neurons.\textsuperscript{61} In another persistent infection caused by Borna virus, virus-expressing neurons may be recognized even in the absence of inflammation,\textsuperscript{62} further indicating that neurons play an active role in immune recognition of viruses and stimulate CD8 T-cell responses.\textsuperscript{62}

Collectively, these data illustrate that neurons: i) are integrated into a complex neuroimmunological network that acts to protect against persisting pathogens, ii) may attract CD8 T cells, and iii) may directly interact with CD8 T cells via MHC class I antigens and Fas/FasL.

\section*{C. Effector Cells of (Auto)Immune Reactions of the CNS}

\subsection*{1. CD8 T Cells Versus CD4 T Cells}

Traditionally, CD4 T cells were considered to be the major effector cells of autoimmune diseases.
of the CNS, particularly in MS and EAE. Until recently, the role of CD8 T cells in autoimmune disorders of the CNS was considered to be of rather low importance; however, this traditional view of CD8 T cells as mere regulators in autoimmune responses has been questioned on the basis of several observations. CD8 T cells are abundantly present in the brain in most inflammatory CNS disorders of both an infectious and an autoimmune nature and numerically dominate over CD4 T cells,63-66 in MS lesions, CD8 T cells outnumber CD4 T cells almost 10-fold.63,67 Clonal expansion of T cells within inflammatory lesions of the brain is more readily observed in the CD8 than in the CD4 T-cell population in MS and in paraneoplastic disorders, which suggests their local antigen-driven activation.64,68,69 In MS, axonal damage within lesions correlates more closely with the number of CD8 T cells and macrophages/microglia than with CD4 T cells. CD8 T cells from mice deficient in T-bet and eomesodermin differentiate excessively into IL17+-secreting cells upon viral infection and develop a devastating IL17+CD8 T-cell-mediated autoinflammatory syndrome.70 In an elegant humanized mouse model for MS,71 two waves of leukocytic infiltration were noticed. In the early wave, CD8 T cells were far in excess of CD4 T cells, while CD4 T cells accounted for 75% of all T cells and were clearly dominant among late-infiltrating T cells. These observations suggested antigen release and epitope spreading during early disease. CD8 T cells appear to be able to initiate the first autoimmune attacks, while disease progression depends on CD4 T cells.

In addition, CD8 T cells may induce oligodendrocyte lysis in vivo as a likely consequence of direct antigen recognition, as demonstrated by adoptive transfer of hemagglutinin-specific CD8 T cells into mice expressing hemagglutinin specifically in oligodendrocytes.72 These data extend and are in line with the observation that myelin-specific CD8 T cells induce severe CNS autoimmunity in mice.71-75 In TMEV infection, autoreactive CD8 T cells have been identified as critical effector cells in autoimmune demyelination. They recognize self-antigen presented by MHC class I molecules on oligodendrocytes, possibly through molecular mimicry between TMEV and host proteins,76 and can kill both virus-infected and uninfected target cells.

Antigen-specific CD8 T cells also contribute to the loss of motor function by disrupting axonal transport, and thus may be responsible for the initiation of axon injury following demyelination.77 In addition to precisely elucidating the role of CD8 T cells in autoimmune demyelination, studies in TMEV infection were the first to provide an experimental link between virus infection and subsequent autoimmunity.78

CD8 T cells can attack astrocytes as well as oligodendrocytes, which was demonstrated in a transgenic mouse mutant in which astrocytes expressed hemagglutinin.79 In this model, the transfer of activated MHC class I-restricted, hemagglutinin-reactive T cells induced monophasic brain inflammation with selective destruction of astrocytes. Specific destruction in the absence of bystander damage appeared to directly reflect T-cell-mediated cytotoxicity. Although human disease resulting from autoimmune damage to astrocytes exclusively has not yet been reported, one may still envision its existence; because neurological symptoms were not reported in mice expressing hemagglutinin transgenically in astrocytes, autoimmune destruction of astrocytes may well occur in humans and may also remain clinically asymptomatic. This may, at least in part, be explained by a relatively strong regenerative capacity of astrocytes. Interestingly, apoptosis of astrocytes was recently observed in human Rasmussen’s encephalitis and was shown to be mediated by CD8 T cells.80

In any discussion of the role of CD8 T cells in autoimmune diseases of the CNS, it should be stressed that, in addition to disease-promoting TC1 and TC17 cells, regulatory CD8 T cells have also been identified.81 These Qa-1-restricted CD8 T cells suppress autoimmune responses in EAE, and gene deletion of Qa-1 exacerbates EAE due to the lack of inhibition of CD4 T cells by regulatory CD8 T cells.

2. CD8 T-Cell-Mediated Neurological Diseases Due to Targeting Neurons

In humans, clinically relevant neurological autoimmune disorders in which neurons are the target of the autoimmune attack mediated by CD8 T cells are paraneoplastic disorders82-84 and Rasmussen’s encephalitis.85
Paraneoplastic diseases include various syndromes such as paraneoplastic encephalomyelitis (anti-Hu syndrome), which may manifest as limbic encephalitis, brain stem encephalitis, or cerebellar degeneration (anti-Yo syndrome). In these disorders, cells of small-cell lung, breast, and ovarian cancers ectopically express antigens that are normally restricted to neurons, and therefore have been termed onconeural antigens. The immune response is intended to suppress tumor growth, which is often effective because the primary tumor may escape detection, while patients clinically suffer from neurological symptoms due to autoimmune intracerebral inflammation and the subsequent loss of the neuronal population expressing the onconeural antigen. Disease is suggested to be CD8 T-cell mediated. Pathogenetically, a model of cross-presentation has been proposed in which the antigen from apoptotic tumor cells is taken up by dendritic cells in peripheral organs and presented to CD8 T cells in lymph nodes. Subsequently, T cells home to the brain, where they attack neurons and cause neuronal apoptosis. This model is strongly supported by the observation of an oligoclonal expansion of the CD8 T cells in the CNS. In this scenario, the uptake of apoptotic neurons by antigen-presenting cells in the brain may further amplify the autoimmune response. The preferential localization of the inflammatory reaction, which is characterized by perivascular lymphocytic infiltrates and an activation of microglia in the mediotemporal structures of the brain, cerebellum, brain stem, and anterior horn cells of the spinal cord, is remarkable (Fig. 2a–c).

Rasmussen’s encephalitis, a rare form of childhood epilepsy with intractable seizures, has traditionally been considered to be an autoimmune humoral disorder caused by autoantibodies directed against subunit 3 of the ionotropic glutamate receptor. However, recent data provided strong evidence for a CD8 T-cell-mediated disorder with targeted destruction of neurons by granzyme-B-expressing CD8 T-cells lying in direct apposition to MHC class I+ neurons (Fig. 3a,b). While the particular (auto)antigen recognized by these CD8 T cells still remains to be identified, clonal expansions of CD8 T cells have been detected in the peripheral blood of patients, and may persist for at least 1 to 2 years. Furthermore, complementarity-determining region 3 spectratyping suggested similarities in the antigen specificity of the expanded T-cell clones between different patients, indicating a common antigen. The strong clonal expansion in the peripheral CD8 T-cell compartment sug-
gested an ongoing CD8-mediated autoimmune response instead of a random attraction of T cells as part of a secondary immune response. Thus, an ongoing process triggering survival or continuous activation of pathogenic CD8 T-cell clones may be induced by exposure to CNS autoantigen(s). The autoantigen may not be restricted to neurons, but may also be expressed by astrocytes, which also undergo CD8 T-cell-induced apoptosis in Rasmussen's encephalitis.80 Alternatively, activation may be caused by a persisting virus that may have infected both neurons and astrocytes. Interestingly, a viral etiology was suggested by Rasmussen in 1958.89 In fact, there are similarities between Rasmussen's encephalitis and Russian spring-summer meningoencephalitis, a flavivirus-induced meningoencephalitis.90 In addition, EBV and cytomegalovirus have also been implicated in several cases91,92; however, these data await confirmation and a causative agent still remains to be identified.

In addition to disorders in which neurons are the primary target of an (auto)immune attack, they may also be damaged secondarily during an immune reaction. Recently, the concept of “cumulative autoimmunity” has been proposed based on the observation that myelin oligodendrocyte glycoprotein (MOG)-T-cell-receptor transgenic mice unexpectedly developed EAE spontaneously in the absence of MOG due to recognition of a neuronal peptide fragment of the medium-sized neurofilament by MOG35-55-specific CD4 T cells.93 Similar observations have so far not been reported for CD8 T cells. Furthermore, direct inflammatory injury to lower motor neurons in the lumbar spinal cord has been observed in MS and EAE patients.94 CD3 T cells were shown to invade the spinal cord early in disease and induce neuronal apoptosis, and dying neurons were in close contact with T cells containing the death-ligand TRAIL (TNF-related apoptosis-inducing ligand), a mediator of neuronal damage.

D. Role of Infections in Autoimmune Responses Against Neurons: The Model of Ovalbumin-Expressing Neurons and L. monocytogenes

In order to define the conditions under which a clinically symptomatic autoimmune reaction against CNS neurons is induced, and to define the role of infectious pathogens in CNS autoimmune responses, our group has developed a transgenic mouse model (NSE-OVA) in which neurons express ovalbumin (OVA) with defined CD8 T-cell epitopes.18 Using this model, we studied the reactions to an infection with the facultative intracellular bacterium L. monocytogenes.

L. monocytogenes is an interesting organism to use for this because it may cause not only a systemic (i.e. hepatic and splenic) infection, but also serious and even fatal CNS disease in humans. L. monocytogenes targets neurons, ependymal cells, and plexus choroid epithelial cells. Control and elimination of bacteria and survival of the patient requires both CD4 and CD8 T cells.38,42

Upon intracerebral infection with attenuated Listeria, which lacked the virulence-associated actA gene and also expressed ovalbumin (ΔactA OVA L. monocytogenes), NSE-OVA mice cleared L. monocytogenes from the brain up to d 5 postinfection. Starting at d 3 postinfection, up to d 7 postinfection, 100% of mice had devel-
oped a neurological syndrome characterized by coordination disturbance, abnormal gait, hind limb ataxia, hind limb weakness, irritability, and tremor. Interestingly, development of the disease required the presence of the pathogenetic antigen in the brain, because mice with an exclusively extracerebral, systemic listeriosis did not develop neurological symptoms. The neurological syndrome was abolished by depletion of CD8 cells, but not CD4 T cells, indicating a CD8 T-cell-mediated autoimmune response. An important and critical clinical aspect of these experiments is that the neurological disease was initiated exclusively by the infection without the addition of large numbers of T-cell receptor-transgenic, OVA-specific CD8 T cells. This is in marked contrast to other models of autoimmune diseases, which require the transfer of T-cell receptor-transgenic T cells in order to induce an autoimmune response against an antigen transgenically expressed on resident brain cells. Nevertheless, an adoptive transfer of OVA\textsubscript{257-264}\textsuperscript{-specific OTI CD8 T cells significantly aggravated neurological disease in the NSE-OVA model.

Neurological symptoms were attributed to apoptosis of CNS neurons, mainly in the spinal cord, the neocortex, and the hippocampus. The close apposition of OVA-specific OTI CD8 T cells with OVA-expressing neurons, which had up-regulated components of the MHC class I complex, further substantiated the hypothesis of CD8 T-cell-mediated neuronal damage (Fig. 4a,b). However, the precise mechanism of how OVA\textsubscript{257-264}\textsuperscript{-specific CD8 T cells kill neurons in this infection-related autoimmune CNS disorder still remains to be identified. The preferential, although not exclusive, targeting of spinal cord motor neurons, with up to 28.9% of anterior horn spinal motor neurons undergoing apoptosis, was remarkable. This observation may support the notion that during immune responses, when T cells recognize their cognate antigen on CNS neurons, their homing to the spinal cord is increased compared with the brain. Such a preferential homing of T cells to the spinal cord has also been observed under physiological conditions and is in accordance with the concept of a compartmentalization of immune privilege in the CNS, which facilitates preferential homing to less-privileged anatomic structures.\textsuperscript{26}

Extracerebral, systemic infections caused by a pathogen related to or similar to the neuronal autoantigen may further modify the intracerebral autoimmune response. This is illustrated by a significant aggravation of the neurologic disease of NSE-OVA mice by a systemic (intraperitoneal) infection preceding intracerebral infection. Aggravation of disease was due to an increased proliferation of OVA\textsubscript{257-264}\textsuperscript{-specific CD8 T cells in the periphery, followed by the recruitment of increased numbers of these OVA\textsubscript{257-264}\textsuperscript{-specific CD8 T cells to the brains of NSE-OVA mice.

The microenvironment of the CNS may also play a role in determining the resulting immune response against neurons. Infection of the brain with attenuated \textit{L. monocytogenes}, although clinically asymptomatic, induced a local proinflammatory
environment that included the recruitment of macrophages and activation of microglial cells with an up-regulation of MHC class I and II antigens. Since local antigen-presenting cells may be required for an induction of autoimmune CNS disorders by molecular mimicry,95 activated microglia may also contribute to a further activation of OVA\textsubscript{257-264}-specific CD8 T cells. However, it is unlikely that they are sufficient to induce the OVA\textsubscript{257-264}-specific CD8 T-cell response, which may require induction by dendritic cells in lymphatic organs. This concept is in accordance with observations in the model of LCMV-induced molecular mimicry in the CNS, in which genes of LCMV were expressed in oligodendrocytes of the brain. Neither expression of the transgene alone nor systemic LCMV infection caused demyelination, resulting only in an infiltration of the brain parenchyma by CD4 and CD8 T cells and an activation of microglia, which did not cause disease.17 In contrast, further viral infection of the CNS with an unrelated virus caused demyelination.17 Thus, under these conditions, the autoimmune reaction against oligodendrocytes was not antigen specific.

### E. Conclusion and Outlook

Ample clinical and experimental observations suggest that autoimmune reactions in the CNS mediated by CD8 T cells can target a broad range of important cell populations. There is evidence for the concept of molecular mimicry playing an important role in this scenario, while bystander activation of T cells appears to be of minor relevance. In addition to oligodendrocytes and astrocytes, neurons are also targeted by the autoimmune response, as illustrated by various clinical disorders such as paraneoplastic diseases and Rasmussen’s encephalitis. Remarkably, in Rasmussen’s encephalitis, neurons as well as astrocytes seem to be targeted by cytotoxic CD8 T cells in an antigen-specific manner.80 This important observation indicates that the in vivo human situation is much more complex than was thought previously, as evidenced by the recent description of “immunological self-mimicry.” Transgenic mice expressing a myelin oligodendrocyte glycoprotein-specific T-cell receptor developed EAE in the absence of myelin oligodendrocyte glycoprotein. In this mutant, CD4 T cells mounted an autoimmune response against two independent target autoantigens in the same tissue, the myelin oligodendrocyte glycoprotein expressed by oligodendrocytes and neurofilament-M, a cytoskeletal protein expressed by neurons.93 Recognition of both autoantigens was possible because epitope sequences of the autoantigens shared essential T-cell-receptor contact positions. Thus, more than one autoantigen may be recognized and have a role in spontaneously developing autoimmune diseases.

Interesting and promising animal models that will allow detailed investigation of the pathogenesis of autoimmune intracerebral reactions have been established. Using genes expressed by pathogens such as viruses or bacteria as autoantigens, the role of infections in autoimmune responses in the CNS can be investigated in depth. There appear to be differences with respect to the requirements for inducing an intracerebral autoimmune response, which may be determined both by the nature of the target cell and the pathogen inducing the (auto)immune response. Interestingly, an autoimmune response against neurons required exactly the autoantigen-expressing pathogen,18 while an autoimmune response against oligodendrocytes can already be induced by an unrelated virus. This observation supports the hypothesis that neurons, as the most vulnerable cell population with the lowest regenerative potential, are the most intensely protected. The relevance of the nature of the target cell is also illustrated in a mouse model of either β-gal-expressing astrocytes or β-gal-expressing retinal neurons, respectively. β-gal-specific CD8 T cells efficiently killed both populations; however, antigen-expressing retinal neurons were killed by a mechanism that was substantially delayed and differed from killing of retinal astrocytes.96 It will be important to determine the relative roles of the individual participants of the autoimmune response, including the autoantigen-expressing cell population and the triggering infectious agent. Currently, the prerequisites to be fulfilled by a pathogen in order to trigger autoimmune responses have not been analyzed sufficiently, and this is pathogenetically important because all clinically relevant pathogens have developed sophisticated immune evasion strategies that might also affect the induction of organ-specific autoimmune responses.
There are further important issues to be addressed, including factors protective against autoimmune reactions in the brain, such as the role of both CD4 and CD8 regulatory T cells. In addition to their function as effectors, T cells may also serve neuroprotective functions in autoimmune reactions. Such a dual role has been recognized for encephalitogenic CD4 T cells, which support neuronal survival by the secretion of potent neuroprotective mediators (i.e., NGF, BDNF, NT-3, NT-4/5) in traumatic damage of the optic nerve and spinal cord and simultaneously damage myelin with their TNF and IFN-γ production.\textsuperscript{97,98} Remarkably, the neuroprotective effect was dependent on the antigen-specificity of the T cells. Thus, under certain circumstances, such as the CNS injury optic nerve crush, T-cell autoimmune in the CNS can exert a beneficial effect by protecting against secondary degeneration of neurons. The capacity to inhibit secondary degeneration may therefore be regarded as a “beneficial” autoimmune response, and is mediated by naturally occurring CD4CD25 regulatory T cells that serve to maintain a balance between the ability to manifest an autoimmune response required for neuroprotection and repair and the need to avoid autoimmune disease. Such a dual role of autoimmune cells in the CNS has been shown for encephalitogenic CD4 T cells, but the identification of regulatory CD8 T cells in EAE\textsuperscript{81} indicates that CD8 T cells may exert protective functions in autoimmune disorders of the CNS.

Experimental animal models are a valuable and useful tool with which to precisely dissect the autoimmune response against an antigen—expressing target cell. With respect to human diseases of the nervous system, in which molecular mimicry may be pathogenetically relevant, the identification of both the underlying (auto)antigen(s) as well as the causative pathogen is a pending challenge.

ACKNOWLEDGEMENTS

The author’s own work is supported by the Deutsche Forschungsgemeinschaft (De 485/8–2).

REFERENCES

14. Croxford JL, Olson JK, Anger HA, Miller SD. Initiation and exacerbation of autoimmune demyelination of the central nervous system via virus-induced molecular mimicry: implications for the pathogenesis of
Role of CD8 T-Cell-Mediated Autoimmune Diseases of the Central Nervous System


56. Grosjean MB, Lenzlinger PM, Stahel PF, Yatsiv I, Shohami E, Trenzt O, Kossmann T, Morganti-Kossmann MC. Immunohistochemical characterization of Fas (CD95) and Fas Ligand (FasL/CD95L) expression in the injured brain: relationship with neuronal cell death and inflammatory mediators. Histol Histopathol. 2007 Mar;22(3):235–50.


Critical Reviews™ in Immunology


Genetic Control of D_H Reading Frame and Its Effect on B-Cell Development and Antigen-Specific Antibody Production

Harry W. Schroeder, Jr.*, Michael Zemlin², Mohamed Khass³, Huan H. Nguyen⁴, & Robert L. Schelonka⁵

¹Division of Clinical Immunology and Rheumatology, Departments of Medicine, Microbiology, and Genetics, University of Alabama at Birmingham, Birmingham, AL; ²Department of Pediatrics, Philipps-University Marburg, Marburg, Germany; ³Department of Microbiology, University of Alabama at Birmingham, Birmingham, AL; ⁴Principal Investigator, Viral Immunology Laboratory, International Vaccine Institute, Seoul, Korea; ⁵Division of Neonatology, Department of Pediatrics, University of Oregon Health Sciences Center, Portland, OR

*Address all correspondence to Harry W. Schroeder, Jr., MD, PhD, Division of Clinical Immunology and Rheumatology, Departments of Medicine, Microbiology, and Genetics, University of Alabama at Birmingham, SHEL 176, 1530 3rd Ave S., Birmingham, AL 35294-2182; Tel.: 205-934-6826; Fax: 205-975-6352; Email: hwsj@uab.edu.

ABSTRACT: The power of the adaptive immune system to identify novel antigens depends on the ability of lymphocytes to create antigen receptors with diverse antigen-binding sites. For immunoglobulins, CDR (complementarity-determining region)-H3 lies at the center of the antigen-binding site, where it often plays a key role in antigen binding. It is created de novo by VDJ rearrangement and is thus the focus for rearrangement-dependent diversity. CDR-H3 is biased for the inclusion of tyrosine. In seeking to identify the mechanisms controlling CDR-H3 amino acid content, we observed that the coding sequence of D_H gene segments demonstrate conservation of reading frame (RF)-specific sequence motifs, with RF1 enriched for tyrosine and depleted of hydrophobic and charged amino acids. Use of D_H RF1 in functional VDJ transcripts is preferred from the earliest stages of B-cell development, “pushing” CDR-H3 to include specific categories of tyrosine-enriched antigen-binding sites. With development and maturation, the composition of the CDR-H3 repertoire appears to be “pulled” into a more refined specific range. Forcing the use of alternative D_H RFs by means of gene targeting alters the expressed repertoire, enriching alternative sequence categories. This change in the repertoire variably affects antibody production and the development of specific B-cell subsets.

KEY WORDS: immunoglobulin, diversity gene segment, antibody repertoire, B-cell development

I. INTRODUCTION

Immunoglobulin (Ig), the B-cell antigen receptor (BCR), is a heterodimeric molecule composed of two heavy (H) and two light (L) chains.¹⁻³ Each H and L chain consists of a variable (V) domain, which binds antigen, and one to four constant (C) domains, which carry out the effector function of that chain. Diversity is asymmetrically distributed within the V domain, with each V containing of three intervals of hypervariability called complementarity-determining regions (CDRs) and four intervals of conserved sequence termed framework regions (FRs) (Fig. 1). The four FRs of

ABBREVIATIONS

BCR, B-cell antigen receptor; CDR, complementarity-determining region; DEX, α(1+3)dextran; FR, framework region; Ig, immunoglobulin; MZ, marginal zone; RF, reading frame; TT, tetanus toxin

Received: 11/01/2009 Accepted: 2/26/2010

1040-8401/10/$35.00 © 2010 by Begell House, Inc.
the H chain and the four FRs of the L chain fold to form the scaffold that brings together the three H-chain and the three L-chain CDRs to create the antigen-binding site, as classically defined.

Evolutionary comparisons of Ig sequences within and across species have shown that the three-dimensional structure of the V domain represents a gradient of diversity. The hydrophobic core of the V domain, which consists of FR2 and FR4, is highly conserved. The sequence of FR1 is also highly conserved. FR1s can take three different basic shapes that identify the evolutionary clan of origin. V gene segment family identity is determined by the sequence of FR3. There are 16 basic family structures in the mouse, and seven in humans. The FR3s border the antigen-binding site, both supporting and confining the shape of CDR1 and CDR2. CDR-H1, CDR-H2, CDR-L1, and CDR-L2, are entirely encoded by their respective V gene segments and are initially limited to the germline sequence. CDR-L3 and CDR-H3 are created de novo by V<sub>L</sub>→J<sub>L</sub> and V<sub>H</sub>→D<sub>H</sub>→J<sub>H</sub> joining, respectively.

Although there can be great variation in the sequence and size of these five CDRs (1, H2, L1, L2, and L3), they form a rather small set of main-chain conformations that are termed canonical structures. Each such structure is determined by the size of the loop and by the presence of certain residues at key positions in both the loop and framework regions. For example, three canonical structure types have been identified for CDR-H1,

**FIGURE 1.** CDR-H3 plays a key role in antigen-binding site diversity. Top: The variable domains of the L and H chains are created by VJ joining, and by VDJ joining and N addition, respectively. Due to the inclusion of a D gene segment, the opportunity to introduce two sets of N nucleotide additions, and the greater flexibility in length and sequence composition, the CDR-H3 interval is the most diverse portion of the pre-immune repertoire (for review, see Shroeder et al., 1998<sup>21</sup>). Bottom: A cartoon of the classic antigen-binding site as seen head-on. Due to its central location, most bound antigens will interact with CDR-H3, including its D<sub>H</sub> component.
four for CDR-H2, five for CDR-L1, one for CDR-L2, and five for CDR-L3.\textsuperscript{6,7,10} Assuming random assortment, we would expect the repertoire to contain 300 different combinations of these canonical structures.\textsuperscript{11} However, only 10 of these combinations account for seven-eighths of human and mouse Fab sequences. Thus, by both sequence and structure, the diversity provided by these five germline-encoded CDRs is even more distinctly finite than was first appreciated, even prior to antigen-driven selection.

Due to the inclusion of a diversity (D) gene segment and the addition of non-germline encoded nucleotides (N regions), CDR-H3 is by far the most variable of the six CDRs (Fig. 1). Enhanced diversity and a central position within the antigen-binding site permits CDR-H3 to often play the most critical role in antigen recognition and binding.\textsuperscript{1,2,12} It is for this reason that CDR-H3 has become a major focus of our studies.

### A. Defining a “Normal” Range for CDR-H3 Diversity

Although highly variable, our comparisons of Ig repertoires between and within species had led us to the hypothesis that young adults might be programmed to express a preferred range of CDR-H3 sequences and structures. The potential diversity of CDR-H3 is so great that it might seem presumptuous to expect that we could use economical methods to identify such conserved features. However, our comparative studies revealed molecular characteristics of CDR-H3 repertoires that appeared to permit recognition of categorical restrictions in diversity after examination of as few as 100 sequences per developmental stage. These characteristics included V, D, and J gene segment usage, the extent of N addition and the length, global amino acid content, and average hydrophobicity of CDR-H3 (Fig. 2).

#### B. Development of the CDR-H3 Repertoire

Construction of CDR-H3 begins early in B-cell progenitors. Indeed, the various defined stages of B-cell development can be viewed, in part, as transitions through a series of checkpoints that test the assembly and function of CDR-H3.\textsuperscript{13-18} In humans, the selection during B-cell development is associated with a reduction in the mean length of the expressed CDR-H3 repertoire,\textsuperscript{19} as well as in decreased frequency of highly charged or hydrophobic sequences.\textsuperscript{20-22}

---

**FIGURE 2.** Deconstruction and analysis of CDR-H3. In this hypothetical sequence, the location of CDR-H3, the CDR-H3 loop, and boundaries of FRs 3 and 4 are shown. Kabat and IMGT\textsuperscript{1,72} (1:72) number designations for the TGT codon, which marks the terminus of framework 3, and the TGG, which marks the beginning of framework 4, are identified. Here, the CDR-3 loop has been evaluated for the distribution of individual amino acids and average Kyte-Doolittle hydrophobicity (73;74). Amino acids at the extreme (arginine and isoleucine) have been included to demonstrate the range of the hydrophobicity index. The normalized average hydrophobicity of this CDR-H3 loop is –0.24. This CDR-H3 has also been evaluated for V\textsubscript{H}, D\textsubscript{H}, and J\textsubscript{H} usage, P junctions, N addition, and the length of CDR-H3 in codons. A single palindromic (P) nucleotide flanks the V\textsubscript{H} sequence. D\textsubscript{H} DFL16.1 sequence is flanked by three nucleotides of N addition on each side. To facilitate analysis, we have color-coded our data in this and other figures in this application to report relative hydrophobicity. Blue reflects hydrophobicity, green represents neutrality with or without hydrophilicity, and red is used for charge.
In order to gain insight into the mechanisms that regulate the antibody repertoire, to determine when during development constraints on CDR-H3 composition are imposed, and to establish the extent to which murine development resembles that of humans, we conducted a detailed examination of CDR-H3 repertoire development in BALB/c mice. We used the scheme of Hardy to sort bone marrow B-lineage cells into progenitor, immature, and mature B-cell fractions. We chose to look at RNA message because this is most representative of the expressed, and thus functional, Ig repertoire. We cloned, sequenced, and deconstructed the CDR-H3 component of VH7183DJCμ transcripts. Subsequently, we used the same cloning techniques to examine CDR-H3 repertoire development in the spleen, focusing on splenic T1 (Loder), marginal zone (MZ), and follicular subsets. We concentrated on the VH7183 family because its germline complement in IgH alleles had been well-defined, it represents a manageable 10% of the active repertoire, patterns of VH7183 utilization during ontogeny and development have been well-established, and it contributes to both self and non-self reactivities.

In wild-type BALB/c mice, we found that the variance in the studied parameters in both spleen and bone marrow decreased as the developing B cells passed through successive differentiation checkpoints (Fig. 3). As in our previous studies in humans, we found that the distribution

**FIGURE 3.** Distribution of CDR-H3 charge in VDJCμ transcripts of wild-type BALB/c mice isolated from phenotypically defined bone marrow and spleen B-cell populations as assessed by reference to a normalized Kyte-Doolittle scale (75,76). Prevalence is reported as the percentage of the sequenced population of unique, in-frame, open transcripts from each B-lineage subset. To facilitate visualization of the change in variance of the distribution, the vertical lines mark the apparent normal boundaries beyond which it appears to be difficult to transition into fraction F.
of gene segment usage, lengths, global amino acid content, and average hydrophobicity exhibited a specific, controlled distribution at the earliest stage of B-cell development evaluated (fraction B), which is prior to the surface expression of immunoglobulin. We observed a consistent enrichment for tyrosine and glycine in CDR-H3, and an apparent selection against both positively charged and hydrophobic amino acids (Fig. 4, bottom). In cross-species studies, we found that the bias for tyrosine and glycine in CDR-H3 was common to all jawed vertebrates (Table 1).25

Geneticists and evolutionary biologists distinguish between selection at the species level, which reflects reproductive fitness and is thus targeted to the germline in order to be transmitted from parent to child, and selection at the level of the individual cell, which reflects fitness in the local environment of that cell and is typically not transmitted to the child. The former is referred to as natural selection and is the operating principle in the evolution of germline sequence content. The latter is referred to as somatic selection, with changes that may be transmitted from progenitor to daughter cell, but will not be transmitted to the next generation of the species. Thus, the conservation of CDR-H3 tyrosine content suggested to us that CDR-H3 sequence content might be the product of natural selection of germline sequence, as well as the clonal somatic selection that operates in the individual in response to local antigenic exposure. In support of this hypothesis, our comparative studies revealed that the preference for tyrosine in the CDR-H3 repertoire of adult B cells mirrored the preferential use of D_{H} gene segments with tyrosine-enriched reading frame 1 (RF1) (Fig. 5).

These observations led us to the hypothesis that natural selection of the D_{H} germline repertoire

FIGURE 4. D-limited mice express polyclonal, altered CDR-H3 repertoires. Prevalence is reported as the percent of the sequenced population of unique, in-frame, open transcripts from each B-lineage fraction. (Right) Distribution of CDR-H3 average hydrophobicity in VDJC_{µ} transcripts from CD19^{+}IgM^{+}IgD^{+} mature, recirculating bone marrow B cells from homozygous ΔD-DFL, ΔD-D_{µ}FS, ΔD-iD, and wild-type (wt) mice. The normalized Kyte-Doolittle hydrophobicity scale (74) has been used to calculate average hydrophobicity. To facilitate visualization of the change in distribution, the vertical lines mark the preferred range average hydrophobicity observed in wild-type fraction F (Fig. 3).23 Left: Distribution of amino acids in the CDR-H3 loop as a function of B-cell development in the same strains of mice. Amino acids are arranged by polarity from arginine (left) to isoleucine (right). The number of sequences per B-cell fraction is shown on the far left.
might play a key role in controlling the global composition of CDR-H3 and thus of the (final) antibody repertoire.

II. GENETIC CONTROL OF THE CDR-H3 REPertoire

In order to test the role of germline control of the D\textsubscript{H} sequence on regulating the composition of CDR-H3, we enlisted the aid of Dr. Klaus Rajewsky to create what we have come to call D-altered, or D-limited, mice (Fig. 6). We created a targeting construct that allows the introduction of altered D\textsubscript{H} sequence into the D\textsubscript{H} locus at the 5' position by means of homologous recombination. We introduce loxP sites downstream of the altered D\textsubscript{H} and just upstream of the J\textsubscript{H} locus. Either in vivo or in vitro cre-mediated deletion then creates an IgH allele that contains only the altered D\textsubscript{H}. Although deletion and manipulation of the D\textsubscript{H} locus have occurred, it must be emphasized that with the rest of the IgH locus is maintained in its normal, germline form. It contains a normal complement of V\textsubscript{H}, J\textsubscript{H}, and C\textsubscript{H} exons. The process of rearrangement occurs in the expected manner at the expected times in development. Following VDJ rearrangement, the amino acid contribution to CDR-H3 of the altered coding sequence of the

<table>
<thead>
<tr>
<th>TABLE 1: Representation of Amino Acids by D\textsubscript{H} Reading Frame is Non-Random and Conserved*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species</td>
</tr>
<tr>
<td>Gene Segment &amp; RF</td>
</tr>
<tr>
<td>RF by Deletion</td>
</tr>
<tr>
<td>RF1 (Hydrophilic)</td>
</tr>
<tr>
<td>RF2 (Hydrophobic)</td>
</tr>
<tr>
<td>RF3 (Hydrophobic &amp; Termination)</td>
</tr>
<tr>
<td>RF by Inversion</td>
</tr>
<tr>
<td>i-RF1 (Charged)</td>
</tr>
<tr>
<td>i-RF2 (Hydrophobic)</td>
</tr>
<tr>
<td>i-RF3 (Hydrophobic &amp; Hermination)</td>
</tr>
</tbody>
</table>

RF: reading frame; Avg HP: average hydropathicity
*Shown are the amino acid sequences of each of the six RFs for the D\textsubscript{H}1 from *Heterodontus* (Litman),\textsuperscript{25} DFL16.1 from mouse,\textsuperscript{26} and D3-22 from human.\textsuperscript{27} The average hydropathicity of each RF has been calculated as described below. In all three species, one RF is employed preferentially (RF1 by deletion) such that the HCDR3 is conserved to be slightly hydrophilic and enriched for aromatic amino acids.

FIGURE 5. BALB/c DH RF1 amino acid sequences.

FIGURE 6. Generation of a D-limited IgH allele.
D_{H} locus is the only signature of the gene targeting that remains in the functional allele. The B cell has access to the normal complement of L chains. Class switching to all isotypes is maintained, as well as the potential for somatic hypermutation. Thus, unlike a classic, single-antibody transgene mutant mouse, altering the sequence of the D_{H} permits progenitor B cells to progress through all of the normal checkpoints of B-cell development while expressing polyclonal antibody repertoires that differ only by the contribution of the altered D_{H}.

Of the 13 functional D_{H} gene segments per haploid genome in BALB/c mice, 12 are derived from the same evolutionary progenitor and share extensive sequence similarity (Fig. 5). This includes a preference for tyrosine in RF1, the preferred RF. (The 13th D_{H} gene segment, DQ52, does not encode tyrosine, but contributes to less than 5% of rearrangements.)

To date, we have access to four mutant IgH alleles (Fig. 7): ADQS52, AD-DFL, AD-DµFS, and AD-iD. The first allele available to us, ADQS52, was created by our collaborators. The DQS52 gene segment has been deleted from this allele, but it retains the other 12 D_{H} elements in germline form. On the other hand, the ΔD in AD-DFL, AD-DµFS, and AD-iD reflects the deletion of 12 of the 13 D_{H} gene segments in the IgH locus. We use DFL to signify that DFL16.1 in germline form has remained behind. We use DµFS to identify a DFL16.1 allele that includes two frame-shift mutations, one of which places RF1 in-frame with the upstream ATG start site, and the other places a termination codon near the terminus of RF1 as well. Finally, we use the

![Genetic Control of D_H Reading Frame](image)

**FIGURE 7.** Four D-limited D_{H} alleles.

**FIGURE 8.** Mutant and control DH sequences. Top: Germline DFL16.1. Middle: A Dµ frame shift in DFL16.1 replaces RF1 Y+G amino acids with RF2 V, T, I, and F (DµFS). Bottom: Replacement of central RF1 Y+G codons with inverted DSP2.2 sequence introduces codons for positively charged R and H as well as polar N in a new RF1.
designated iD to identify the allele in which the
inverted sequence of DSP2.2 has replaced the
central coding sequence of DFL16.1 (Fig. 8). The
ΔD-DFL allele limits the D_{H} locus to the use of
dFL16.1, which, like the majority of the D_{H} gene
segments, is enriched for tyrosine in RF1 (Fig. 7). 
Both the ΔD-DFL and ΔDQ52 alleles contain
fewer D_{H} gene segments than normal, but the
D_{H} that remain(s) encode(s) normal, primarily
tyrosine-enriched RF1 sequence. The ΔDmFS
and ΔD-iD alleles are limited to a single D_{H}, but
unlike ΔD-DFL, the only expressed D_{H} is forced
to preferentially use an alternative RF enriched for
valine (ΔDmFS) or uses the inverted DSP2.2
RF that codes for arginine (ΔD-iD) This review
will focus on mice expressing the ΔD-DFL, ΔD-
mFS, and ΔD-iD alleles.

A. DFL16.1-Specific CDR-H3 Repertoire

DFL16.1 normally contributes to approximately
20% of VDJ rearrangements in BALB/c mice. 
Throughout bone marrow development, ΔD-
DFL B-lineage cells exhibited the same pattern
of CDR-H3 tyrosine and glycine predominance
with limited use of hydrophobic or charged amino
acids that had been previously observed in wild-
type BALB/c mice. In ΔD-DFL mice, the mature,
recirculating B-cell population (fraction F) pattern
of amino acid utilization was equivalent to that
observed in wild-type mice (Fig. 4). However,
ΔD-DFL CDR-H3s contained more neutral
amino acids (serine) and fewer charged amino
acids (aspartic acid and asparagine) than wild-type. 
Re-examination of the RF1 sequences of the D_{H}
gene segments revealed that of the 13 BALB/c
DFL16.1 is the only one that encodes serine.
We compared the prevalence of serine, aspartic
acid, and asparagine in the ΔD-DFL repertoire to
that observed in wild-type DFL16.1-containing
sequences and found it to be similar. The nine
wild-type BALB/c DSP D_{H} gene segments,
which normally constitute the majority of VDJ
joins, can be separated into those that encode
asparagine and those that encode aspartic acid in
RF1. A re-examination of the wild-type CDR-H3
repertoire demonstrated that the relative use of
asparagines and aspartic acid in DSP-containing
V_{\beta}18DJ\mu transcripts was directly related to
whether they used the asparagine-encoding or
the aspartic acid-containing members of the DSP
family. This asymmetric, D_{H}-specific use of serine,
aspartic acid, and asparagine by gene segment was
established at the earliest stages of bone marrow
B-cell development studied (fraction B), and it
remained relatively constant at the subsequent
stages of B-cell development up to and including
fraction F.

We then compared the distribution of CDR-
H3 length and average hydrophobicity of the ΔD-
DFL CDR-H3 repertoire to that of the wild-type
repertoire as a whole, as well as to that portion
of the wild-type sequences that used DFL16.1 and to
the other portion that did not include DFL16.1.
DFL16.1 is six nucleotides longer than DSP gene
segments, and 12 nucleotides longer than DQ52.
Remarkably, DFL16.1 CDR-H3s averaged about
two codons more than DSP-containing CDR-H3s
and four codons longer than DQ52-containing
CDR-H3s. At the earliest stages of B-cell
development examined, fractions B and C, the
average length of ΔD-DFL CDR-H3 sequences
was similar to that of the wild-type DFL16.1-
containing sequences, but different from both the
total wild-type and the non-DFL16.1-containing
CDR-H3s. After B cells successfully pass through
the step of interaction with a surrogate light chain
and begin to rearrange one of the light chain loci
(fraction D), the average length of the ΔD-DFL
CDR-H3 repertoire was similar to both total wild-
type and non-DFL16.1 sequences. In contrast, the
average ΔD-DFL CDR-H3 length differed from
total wild-type and non-DFL16.1 CDR-H3s in
the immature B cell (fraction E) and fraction F
populations. By fraction F, the average ΔD-DFL
CDR-H3 lengths was similar to wild-type DFL
16.1. A similar, although not identical, situation
occurred with regard to average hydrophobicity.
The average hydrophobicity of these loops was
similar to total wild-type and to non-DFL16.1-
containing CDR-H3 in fraction B, achieved
near identity in fraction C, differed in fraction D
(P ≤ 0.01), converged toward the wild-type
DFL16.1 average in fraction E, and then achieved
equivalence with wild-type DFL16.1 in fraction F.

In summary, at the early stages of B-cell
development, the ΔD-DFL repertoire approached
the length or hydrophobicity characteristics of the
wild-type repertoire as a whole, while still main-
taining or being “pushed” by its germline sequence.
to incorporate serine into CDR-H3. Upon successive maturation stages, the AD-DFL repertoire increasingly matched the length and hydrophobicity characteristics of that specific component of the wild-type repertoire normally created by the donor DFL16.1 gene segment, while remaining distinct from that component of the repertoire that does not include DFL16.1. This suggested a critical role for antigen receptor-driven selection, which appears to act by “pulling” the repertoire into a donor D\textsubscript{H}-specific preferred range.

**B. CDR-H3s Enriched for DFL16.1-RF2 Sequence**

In theory, the inclusion of a D gene segment coupled with random insertion of N nucleotides should produce a CDR-H3 repertoire of random diversity. This permissive role of the D has been referred to as D-diversity.\textsuperscript{34} In practice, tyrosine and glycine are heavily overrepresented in CDR-H3, comprising 30% to 40% of the global amino acid content of this hypervariable interval. The tyrosine and glycine content of CDRH-3 reflects the preferred use of D\textsubscript{H} RF1.\textsuperscript{21,22,25,35-40} This RF1 preference treats almost two-thirds of all DJ rearrangements with extreme prejudice, which, at first glance, appears quite wasteful. This led to the suggestion that the expression of D\textsubscript{H} in RF2 might be incompatible with effective antigen recognition and downstream B-cell signaling, a concept referred to as D-disaster.\textsuperscript{34}

Mechanistically, the preference for RF1 in BALB/c mice has been linked to RF-specific properties and sequence motifs that are shared among 12 of the 13 D\textsubscript{H} gene segments. These include a predilection for rearrangement by deletion, the frequent occurrence of stop codons in RF3, which act to reduce the likelihood of creating an open RF among VDJ rearrangements that use RF3; a bias toward rearrangement at sites of sequence microhomology between the 5’ end of the J\textsubscript{H} and the 3’end of the D\textsubscript{H}, which favor rearrangement into RF1; and an ATG start site upstream of RF2 that permits production of a truncated D\textsubscript{\mu} protein.\textsuperscript{37,41-44} The DQ52 gene segment is also preferentially rearranged by deletion, and includes one neutral RF (RF1), one hydrophobic RF (RF2), and one highly charged RF (RF3). However, while RF1 uses glycine, so do RF2 and RF3, and none of the three RFs encode tyrosine. DQ52 also lacks an upstream ATG start site, its 3’ terminus does not share extensive sequence homology with the 5’ termini of the various J\textsubscript{H}, and it encodes a termination codon at the 5’ end of RF1. This DQ52 gene segment, which contributes to less than 5% of the adult repertoire, demonstrates more random RF usage.

Transgenic studies have shown that the bias against use of RF2 in DFL and DSP gene segments can be released when pre-B cells are no longer able to produce membrane-bound D\textsubscript{\mu} protein.\textsuperscript{45} This suggested that D\textsubscript{\mu} H-chain protein could engage the mechanisms of allelic exclusion, thereby inhibiting subsequent V→DJ rearrangement. However, the extent to which the bias for tyrosine in CDR-H3 reflected genetic control of D\textsubscript{H} RF rearrangement preferences was unclear. Also unclear was whether somatic selection during B-cell development would be able to adjust the repertoire to avoid the use of RF2-encoded amino acids\textsuperscript{34} should the use of RF2 be increased by genetic means.

In order to address the role of D\textsubscript{H} sequence in regulating RF usage and CDR-H3 amino acid content, we introduced two frame-shift mutations into DFL16.1 and then forced the use of this new D\textsubscript{H} to create the AD-D\textsubscript{\mu}F8 allele (Fig. 8).\textsuperscript{46} The first of the two frame-shift mutations placed the D\textsubscript{\mu} open RF ATG in frame with RF2 instead of RF1. The second shifted the region of D\textsubscript{H}J\textsubscript{H} microhomology from RF2 to RF1, and at the same time shifted one of the two TAG termination codons from RF3 to RF1. These two frame-shift mutations flipped the normal 3:1 preference for RF1 among progenitor B cells to a 3:1 preference for RF2. This 3:1 ratio was largely maintained throughout B-cell development in the bone marrow, including recirculating IgM\textsuperscript{+}IgD\textsuperscript{−} fraction F. Thus, germine control of the repertoire again played a deciding role, providing the “push” in the “push–pull” described above. The result was a repertoire that was enriched for valine, as well as other RF2-encoded hydrophobic amino acids, with a compensatory decrease in the use of tyrosine (Fig. 4).

Although somatic selection did not have the power to recreate the normal preference for tyrosine, we again witnessed the effect of somatic selection, or antigen receptor-driven “pull,” to create a DFL16.1 motif-specific RF1 repertoire.
When we compared CDR-H3 composition by RF, we found that the ΔD-DμFS RF2 repertoire generally matched that generated by DFL16.1 RF2 in both wild-type and ΔD-DFL mice.

More strikingly, RF1-using CDR-H3s from fraction F-type B cells in ΔD-DμFS mice generated a neutral CDR-H3 loop repertoire with a pattern of amino acid usage and an average length that was similar to that obtained from the RF1-generated repertoire from controls. This was unexpected, because when we created the second frame-shift mutation, we made TAG the penultimate 3′ codon of the DμFS RF1. Use of this RF in a functional sequence thus requires that the mutant Dm B cells undergo a minimum loss of five nucleotides. This was borne out in practice, with productive in-frame Vα7183-D-J-C μ transcripts from ΔD-DμFS fraction B cells losing an average of eight 3′ terminal nucleotides versus an average loss of only four 3′ terminal nucleotides among DFL16.1-containing transcripts from the controls (P < 0.001). Because the extent of 5′ loss among the ΔD-DμFS fraction B cells was statistically indistinguishable from controls, the increased loss of 3′ nucleotides led to ΔD-DμFS RF1 contributing, on average, approximately four fewer germ line encoded nucleotides to CDR-H3 (P < 0.05). As a result, the average length of CDR-H3 in fraction B was almost one codon shorter than controls. With development, however, the contribution of germine Dm sequence increased. This effect was most notable among B cells from fraction F, where the average loss of 3′ nucleotides had dropped from eight to seven; and, more importantly, where the average loss of 5′ nucleotides dropped precipitously from six to two (P < 0.05). The effect was to create a mature ΔD-DμFS RF1 repertoire that maintained the same relative contribution of germine Dm sequence as wild-type DFL16.1 RF1 in CDR–H3 intervals of the same average length, thus completely compensating for the loss of 3′ tyrosine with the inclusion of a 5′ tyrosine encoded by the 5′ terminus of the Dm (Fig. 8).

In summary, while the absolute sequence of the RF1-encoded CDR-H3-centric antigen-binding site repertoire was not recreated, the global amino acid composition of the repertoire in mature B cells appears to have been “pulled” by antigen receptor-driven selection to match that observed in both wild-type and ΔD-DFL-containing CDR-H3s that use DFL16.1 in RF1. Together, these findings not only emphasized the critical role of germline Dm sequence in creating the final range of CDR-H3 diversity, or the “pull,” but also indicated that each Dm RF creates its own preferred CDR-H3 repertoire and that this repertoire is shaped by the “push” of somatic selection to fit into a preferred range of lengths, amino acid content, and average hydrophobicity.

C. CDR-H3 Repertoire Enriched for Arginine and Other Positively Charged Amino Acids

The ΔD-DμFS allele forced mice to use an RF that is expressed less frequently than the one that is normally preferred, but that still contributes to a significant portion of the CDR-H3s that comprise the normal repertoire. Thus, it remained possible that the negative effect of using this less-frequent RF was not sufficient to completely engage somatic selection mechanisms. To further test the relative power of somatic versus natural (i.e., germline driven), we created a fourth ΔHm-altered allele, ΔD-iD, in which the use of an inverted RF sequence was forced. The inverted RF selected encodes charged amino acids, especially arginine. This RF normally contributes to a miniscule portion of the repertoire.

To force use of this RF sequence in the context of the normal mechanisms of RF usage, we created an iD ΔH gene segment in which we replaced the core of DFL16.1 with inverted DSP2.2 in RF1. We preserved 5′ and 3′ terminal nucleotides from the recipient DFL16.1 ΔH thus maintained shared microhomology with VH and JH, respectively. The new iD ΔH gene segment thus replaced central tyrosine, glycine, and serine RF1 codons with arginine, histidine, and aspartagine codons from inverted RF1(i-RF1), while maintaining the 5′ and 3′ terminal sequences of DFL16.1 (Fig. 8).39,47

As in our previous studies, we found that somatic selection could not overcome the global effect of changing the ΔH sequence (Fig. 4). In fraction B, 74% of the sequences used the new arginine-enriched RF1, and in fraction F, 80% used the same RF. Although ΔH inversions were more frequent in ΔD-iD B cells than in the wild-type or ΔD-DFL controls, their prevalence did not increase with development, even though i-RF1 for
iD recapitulates the normally preferred tyrosine-enriched sequence of DSP2.2 RF1. Further, we found no evidence of selection for sequences that had undergone extensive exonucleolytic loss or for those with increased N nucleotide content.

The stability of exonucleolytic loss and N region gain created CDR-H3 repertoires whose average length remained unchanged during development. The preservation of the iD sequence contributed to a predominance of arginine, asparagine, and histidine at all stages of bone marrow repertoire development examined (Fig. 4). Together, these amino acids comprised approximately one-third of the amino acids in the CDR-H3 loop, tripling their contribution to the repertoire compared with controls \( (P < 0.001) \). Conversely, the contribution of tyrosine and glycine to the loop was halved \( (P < 0.001) \). Persistence of the charged amino acids was associated with enrichment for CDR-H3 loops with an average normalized Kyte-Doolittle hydrophobicity value of less than –0.700 (Fig. 4).

Evidence of somatic selection was still obtained, however, thus conforming to the “pull” of presumed antigen receptor-driven pressures. Although highly charged CDR-H3 loops were retained in the mature ΔD-iD B cell repertoire, highly hydrophobic sequences followed the normal pattern of loss during development.\(^{23,40}\) The end result of the selective loss of these highly hydrophobic intervals in ΔD-iD shifted the average hydrophobicity of the CDR-H3 repertoire firmly into the charged range.

Thus, even in this extreme case, the germline sequence of the DH still dictated, or “pushed,” the general outline of the CDR-H3 repertoire, with somatic selection apparently focusing, or “pulling,” the repertoire into a range that is “acceptable” to the organism.

**III. ROLE OF THE CDR-H3 REPertoire IN CONTROLLING B-CELL DEVELOPMENT**

Our evolutionary comparisons had previously indicated evidence of strong natural selection pressure to maintain D\(_H\) sequence content and thus, by extension, CDR-H3 content. Having found that changes in the repertoire were not a barrier to successful B-cell production, we then sought to determine whether the change in the repertoire would have an effect on B-cell development.\(^{39,48,49}\) We compared the absolute numbers of B cells in our D-altered mice with wild-type controls, focusing on key developmental subsets in the bone marrow, spleen, and peritoneal cavity.\(^{40}\) The effect is most easily visualized by illustrating the percent divergence in absolute numbers of B cells in these key subsets between individual D\(_H\) mutants and wild-type littermate controls. A graphic depiction of this data (Fig. 9) enables a quick view of the pattern of impairment or enhancement in the numbers of B cells in each fraction relative to wild-type.

In the bone marrow of our D-limited mice, we observed an increase in the number of fraction B (pro-B cells), equivalence in the number of fraction C (early pre-B cells), and a decrease in both the numbers of fraction D (late pre-B cells) and the numbers of fraction E (immature B cells) irrespective of D\(_H\) sequence. This suggests that the increase in the pro-B-cell population and the decrease in the late pre-B- and immature B-cell subsets were due to the loss of 87 kb of the D\(_H\) locus, rather than the change in the sequence of CDR-H3.

However, once released from the bone marrow, the change in the sequence of CDR-H3 had dramatic effects on B cell numbers.\(^{39,40,48}\) In mice limited to the use of a single, normal DFL16.1 gene segment (ΔD-DFL), the numbers of transitional, MZ, and follicular cells in the spleen; the numbers of B1a, B1b, and B2 cells in the peritoneal cavity; and the numbers of mature IgM\(^+\)IgD\(^+\) fraction F cells in the bone marrow proved statistically indistinguishable from wild-type littermates. Thus, mice limited to 20% of the normal repertoire were able to populate all of the peripheral B-cell niches that we examined and to achieve normal numbers of B cells in these niches. In contrast, in the mice that were forced to increase their use of either hydrophobic or positive charged amino acids in CDR-H3, the numbers of conventional splenic follicular and bone marrow mature fraction F-cell numbers were nearly halved.

The effect of the change in CDR-H3 content on the MZ was of particular interest, because follicular and MZ B cells appear to have different functions. The follicular subset contains the resting precursors of cells that appear most likely to engage in immune responses to T-dependent antigens.
Upon stimulation by antigen, follicular cells can give rise to both primary antibody-forming cells and memory B cells of high affinity. In contrast, and based on surface phenotypic criteria, many MZ B cells appear to exist in a semi-activated state, primed to respond to T-cell-independent challenges. However, when given the proper stimuli, MZ cells can engage in T-dependent responses.

In our studies, normalization of MZ B cell numbers was observed in mice using the ΔD-DµFS D1 allele. In contrast, in mice forced to use arginine-enriched ΔD-iD, MZ B-cell numbers increased by one-third (P < 0.01). The increase in MZ B-cell numbers in the ΔD-iD mice (Fig. 9) suggested to us that the MZ repertoire might be either permissive or even selective for charged sequences. Subsequently, we examined the composition of CDR-H3s cloned from wild-type MZ B cells and found that the repertoire is normally enriched for positively charged amino acids (Fig. 3). These findings provided support for our
hypothesis that the increase in MZ cell numbers in ΔD-iD mice simply reflected an increase in the number of B cells bearing BCRs that were acceptable for entry into and survival in this compartment.

Studies from a number of investigators indicate that the MZ is often enriched for self- or polyreactive B cells, including those with potentially pathogenic polyreactivity.53-56 A relative increase in the numbers of activated, self-reactive MZ B cells has been seen in several autoreactive states57-60, however, the functional role of these self-reactive MZ B cells in healthy individuals remains unclear. An excess of charged amino acids in CDR-H3 has been associated with pathogenic self-reactivity, especially to DNA.61-64 In this light, it should be noted that B cells expressing anti-dsDNA reactivity have been shown to be excluded from the follicles.65

IV. ROLE OF CONTROL OF THE CDR-H3 REPERTOIRE ON ANTIBODY PRODUCTION AND RESPONSES TO ANTGEN

In general, we found that the greater the divergence from wild-type, the more truncated the immune response. That is, ΔD-DFL mice were the closest in function to wild-type, followed by ΔD-DμFS, whereas ΔD-iD mice were the most divergent.39,40,48 We began by measuring serum immunoglobulin levels. Homozygous wild-type, ΔD-DFL, and ΔD-DμFS mice expressed equivalent serum levels of IgM, IgA, and IgG, including the four IgG subclasses. However, while the serum concentrations of IgM and IgA were comparable to wild-type, the geometric mean concentration of all four IgG subclasses in the sera of ΔD-iD mice were significantly less than wild-type (P = 0.02, 0.0004, 0.003, and 0.0002, respectively).

In wild-type BALB/c mice, intravenous challenge with α(1→3)dextran (DEX) elicits a T-independent response that is dominated by λ1-light-chain-bearing antibodies that express a diverse range of antigen-binding sites with heterogeneous CDR-H3 sequences.66,67 Seven days after challenge with DEX, we measured the geometric mean of IgM anti-DEX serum levels in homozygous wild-type ΔD-DFL, ΔD-DμFS, and ΔD-iD mice and observed a progressive decline. These results suggested a direct correlation between the extent of divergence from the normal repertoire and the divergence of the host response to this T-independent antigen. In BALB/c, the primary response to the nitrophenylacetyle hapten of NP19-CGG requires T-cell help and contains a large fraction of IgG1 anti-NP antibodies.68 Among those sequences that have been cloned from this population, many incorporate DFL16.1 in RF1. After primary and secondary intraperitoneal challenge with NP19-CGG, the anti-NP IgG response in the ΔD-DFL and ΔD-DμFS mice proved indistinguishable from littermate controls. Conversely, the anti-NP response in ΔD-iD, which requires somatic mechanisms to generate DFL16.1-like sequences, was diminished 3-fold.39

In BALB/c mice, immunization with purified tetanus toxoid elicits a T-dependent response that is dominated by κ-light-chain-bearing antibodies.69 We performed an oral immunization with a recombinant strain of Salmonella that expresses the Tox C fragment of tetanus toxin (TT).70 Unlike the response to DEX or NP, in some cases, repertoire alteration led to an increase in post-challenge titers; in other cases, it led to a decrease, and in yet a third category, there was no change. For example, the IgM anti-tetanus toxoid response doubled in ΔD-DFL mice (P = 0.04); and there was a 4-fold increase in the ΔD-DμFS mice (P = 0.008). IgM responses in the ΔD-iD mice were similar to wild-type. The total IgG anti-TT response in ΔD-DFL mice was slightly increased, primarily due to a 16-fold increase in the IgG3 anti-TT response (P < 0.0001). This pattern was reversed in ΔD-DμFS, in which the IgG anti-TT titer was 16-fold reduced (P = 0.004), with a concomitant 6-fold reduction in IgG1 (P = 0.04) and IgG2a (P = 0.03), and a 34-fold decrease in IgG2b. The IgG3 titer proved equivalent to wild-type. In ΔD-iD mice, the IgG response was approximately 4-fold diminished (P = 0.0004). The IgA response in all three D-altered strains proved indistinguishable from wild-type.

To assess the role of the composition of the CDR-H3 repertoire in response to a viral infection, we immunized homozygous ΔD-DFL and ΔD-iD mice with A/Udorn (H3N2) influenza virus. In both sets of mice, the titer against whole influenza virus was equivalent to wild-type controls. After vaccination, both mutant mice were also found to be equivalently protected against homotypic virus
compared with littermate controls. However, after vaccination with A/Udorn (H3N2), we challenged the mice with the heterologous strain A/pr/8/34 (H1N1), and 40% of the ΔD-iD mice died, whereas all of the ΔD-DFL and the wild-type littermates survived ($P < 0.01$) (Fig. 10). From these data, we concluded that forcing the global Ig CDR-H3 repertoire to use charged CDR-H3s, a “disfavored” category, increases susceptibility to virus infection.

V. CONCLUSION: NATURE VERSUS NURTURE REVISITED

A central focus of debate among students of the adaptive immune response is the relative contribution of germline content versus somatically generated diversity in regulating lymphocyte function. The final composition of this repertoire can be viewed as a compromise between two competing forces. On the one hand, there is pressure to make the repertoire as rich and diverse as possible in order to allow the immune system to recognize novel antigens. On the other hand, the capacity of the genome to encode diversity is distinctly finite, and the production of unnecessary or pathogenic antibodies must be avoided. Although at first glance the CDR-H3 component of the antibody repertoire appears random, our findings suggest that even this most diverse portion of the antibody contains a strong element of predetermination. We have shown that alteration of the germline-controlled CDR-H3 repertoire leads to changes in B-cell development and antibody production. Still to be examined is the extent to which CDR-H3 control influences immune responses to vaccines and pathogens other than influenza virus, as well as the likelihood of developing diseases of immune function, including autoimmune diseases.

FIGURE 10. Mortality after challenge with mouse-adapted heterologous influenza virus. Mice were immunized with influenza strain A/Udorn (H3N2) and then challenged with the heterologous strain A/pr/8/34 (H1N1) at d 0. Values are the percentage of mice that remained alive in each group of 30 immunized homozygous wild-type (wt/wt); 10 immunized homozygous ΔD-DFL (ΔD-DFL/ΔD-DFL); 10 immunized homozygous ΔD-iD (ΔD-iD/ΔD-iD); and 10 naive homozygous wild-type wt/wt mice, respectively, on the given day after challenge.

Critical Reviews™ in Immunology
These issues are under intense investigation in our laboratories.

REFERENCES


Tumor Antigen Presentation by Dendritic Cells

Troels R. Petersen, Nina Dickgreber, & Ian F. Hermans*
Malaghan Institute of Medical Research, Wellington, New Zealand

*Address all correspondence to: Dr Ian F. Hermans, Malaghan Institute of Medical Research, P.O. Box 7060, Wellington, New Zealand, 6012; Tel.: 64-4-499-6914; Fax: 64-4-499-6915; Email: ihermans@malaghan.org.nz.

ABSTRACT: Tumor cells are generally regarded as poor stimulators of naive T cells. In contrast, dendritic cells (DCs) are highly specialized in this function, and are therefore likely to be important intermediaries in the process of stimulating T cell responses to tumors. While providing solid evidence that DCs participate in antitumor immunity has proved difficult, several lines of evidence point in this direction. First, animal models involving bone marrow chimeras have shown that cells of hematopoietic origin are required to elicit T cell responses to whole-tumor vaccines. Second, compared with other cells of hematopoietic origin, DCs are particularly well-equipped to cross-present exogenous antigens to CD8+ T cells, a critical function if intermediary cells are involved. Third, tumor-infiltrating DCs purified from tumor samples have the capacity to cross-present tumor antigens in vitro. Finally, priming of anti-tumor T cell responses can be abrogated in new in vivo models in which DCs can be specifically depleted. It is therefore significant that DCs in cancer patients are often kept in an immature or dysfunctional state, thereby preventing stimulation of tumor-specific T cells. This review describes the different steps required for DCs to elicit T cell responses to tumor-associated antigens, and highlights processes that are amenable to intervention as therapy. We conclude that effective anti-tumor activity may be dependent on the ability to re-program DCs resident in the host, perhaps even when transferred autologous DCs generated ex vivo are used as vaccines. In this context, recruiting the activity of cells of the innate immune system to condition host DCs may help elicit more effective T cell-mediated responses.

KEY WORDS: dendritic cells, antigen presentation, tumor antigens, cross-presentation, NKT cells.

ABBREVIATIONS

APC, antigen-presenting cell; CatS, cathepsin S; CLIP, class II-associated invariant-chain peptide; CLR, C-type lectin receptor; CT, cancer-testis; CTL, cytotoxic T lymphocyte; Cx, connexin 43; DAMP, damage-associated molecular pattern; DC, dendritic cell; DT, diphtheria toxin; DTR, diphtheria toxin receptor; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum-associated protein degradation; ERAP, endoplasmic reticulum-associated aminopeptidase; GM-CSF, granulocyte-macrophage colony-stimulating factor; HMGB, high-mobility group box; HSP, heat-shock protein; IFN, interferon; IL, interleukin; ITAM, immunoglobulin family tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibition motif; KDR, kinase insert domain-containing receptor; LC, Langerhans cell; LCMV-GP, lymphocytic choriomeningitis virus glycoprotein; LOX-1, lectin-like oxidized low-density lipoprotein particle receptor-1; MBL, mannose-binding lectin; MDSC, myeloid-derived suppressor cell; MFG-E8, milk fat globulin-E8; MHC, major histocompatibility complex; NK, natural killer; NKT, NK-like T cells; NLRP3, NOD-like receptor family pyrin domain containing-3 protein; OVA, ovalbumin; PAMP, pathogen-associated molecular pattern; pDC, plasmacytoid DC; PRR, pattern recognition receptor; RAGE, receptor for advanced glycosylation end products; SR-A, class A scavenger receptor; TAA, tumor-associated antigens; TAP, transporter associated with antigen processing; TERT, telomerase reverse transcriptase; TGF, transforming growth factor; TIDC, tumor-infiltrating DC; TNF, tumor necrosis factor; TLR, Toll-like receptor; Treg, regulatory T; TRP, tyrosinase-related protein; TSA, tumor-specific antigen; VEGFR, vascular endothelial growth factor receptor
I. INTRODUCTION

The term “cancer,” which is the Latin word for “crab,” was adopted in ancient times to describe the way in which malignant tumors appear to grasp the tissues they invade. Cancers arise as a result of mutations or epigenetic changes to genes that control cell division, death, and migration, resulting in an abnormality in cellular proliferation associated with life-threatening invasive growth and metastases. Alterations in the function of only a few key genes are necessary to initiate this process, although the subsequent genetic instability permits accumulation of further mutations, and dysregulates the expression of other normal proteins. Analysis of breast and colorectal cancers showed that an average of 90 different mutations accumulate through point mutation, deletion, or chromosomal translocation. In theory, many of these mutations have the potential to create novel targets for immune recognition. Additionally, dysregulated proteins that would otherwise be ignored in normal tissues may also become effective targets for immune attack. Initial studies in mice, together with accumulating clinical experience, indicate that the immune system can indeed recognize and reject tumors. However, these responses are only rarely effective, and it remains a prime focus of immunology research to determine how best to unleash the full power of the immune system to effect cures in cancer patients.

This review will focus on adaptive immune responses to tumor antigens characterized by CD4+ and CD8+ T cells that recognize peptide fragments derived from the mutated or dysregulated proteins presented by major histocompatibility complex (MHC) class I and II molecules. This process initially drives activation of peptide-specific T cells by antigen-presenting cells (APCs), and also renders tumor cells susceptible to attack once T cells have undergone clonal expansion and acquisition of “killer” and “helper” effector functions. Important intermediaries in this process are dendritic cells (DCs), the APC type ultimately responsible for determining when and where T cell activation takes place.

The phagocytic and migratory functions of DCs enables the acquisition of tumor antigens from the tissues, and then presentation of antigenic peptide fragments via MHC molecules to T cells located in the lymphoid tissues. Only when T cells have recognized antigen on DCs, and received appropriate stimulatory signals, will they recirculate to the tumor to exert their effector functions. It is important to note that innate lymphocytes such as natural killer (NK) cells and NK-like T (NKT) cells can also mediate helper and killer functions, although they are not thought to acquire memory. A theme of this review is that effective anti-tumor activity is dependent on the ability of DCs to see tumors as “dangerous,” and to present tumor antigens in a context that recruits the activities of cells of both the innate and adaptive immune systems.

II. TYPES OF TUMOR ANTIGENS

Activated CD8+ T cells with cytotoxic activity (cytotoxic T lymphocytes or CTL) have been isolated from cancer patients and shown to have specific activity against autologous tumors in vitro. The availability of these CTL lines has enabled the identification of the gene products that serve as specific targets in tumor tissue. Because both cellular and humoral (antibody-mediated) responses participate in immune recognition of tumors, it is also possible to use the antibody repertoire in a cancer patient’s serum to identify tumor antigens. A process of “reverse immunology” can also be used to identify tumor antigens. In this process, a candidate tumor antigen is selected based on expression in the tumor relative to normal tissues, and then peptide sequences that can potentially bind defined MHC molecules are predicted by computer algorithms. These strategies, or variants thereof, are currently providing an ever-growing list of suitable targets for T cell-mediated immune responses to tumors. A convenient list of antigens, including epitope structures known to bind specific MHC molecules is provided at http://www.cancerimmunity.org. These antigens can be classified according to their expression and structure, as briefly described in the following sections.
A. Tumor-Specific Antigens

Unique peptide fragments spanning mutations found in tumor tissue, together with idioype-specific peptides from leukemic B or T cells, can be classified as tumor-specific antigens (TSAs). Some of these mutated antigens may be involved in the process of neoplastic transformation itself. For example, mutations in p53 abrogate the regulatory function of this protein on the cell cycle, and therefore contribute to tumor formation. Furthermore, many p53 mutations increase the half-life of the protein, so that the accumulated protein levels provide a large source of mutated peptide for antigen-presentation. Many other TSAs are unique to an individual’s tumor, or are at least restricted to only a few patients, so that immune responses to these antigens are likely to be highly patient specific. Specific T cells of high avidity are likely to be found within the host repertoire to these “neo-antigens,” so that responses to TSA have the potential to be particularly potent.

B. Tumor-Associated Antigens

Another group of antigens are expressed in tumor tissue and only a limited range of normal tissues, and are referred to as tumor-associated antigens (TAA). One particularly useful group of TAA are the “cancer-testis” (CT) antigens, which result from epigenetic changes that drive the expression of gene products normally found in germ cells of healthy adult testis, fetal ovary, or placental trophoblasts. Some limited expression of CT antigens has also been observed in undifferentiated somatic tissue, which, taken together with their expression in germ cells, implies a role for these gene products in embryonic development. Significantly, expression of these antigens in normal tissues is usually restricted to “immune-privileged” sites, where physical barriers and reduced MHC expression protect the tissue from immune attack. Directing immune responses to CT antigens in cancer patients is therefore less likely to be associated with significant autoimmune activity.

A second group of TAAs are the differentiation antigens, which are expressed in tumor tissue and the normal tissue of origin for the malignancy. Examples of differentiation antigens in melanoma include gp100, MART-1, tyrosinase, and tyrosinase-related protein (TRP)-1 and -2, which are all implicated in the normal processes of melanin synthesis or melanosome biogenesis in melanocytes.

A third group of TAAs are the overexpressed antigens, which are expressed in a wide variety of normal tissues, but significantly overexpressed in tumors. The tumor suppressor protein p53 often also falls into this category, because many cancers show abnormally high expression of wild-type protein. Given the expression of p53 in most normal tissues, albeit at low levels, immune responses directed to tumors overexpressing wild-type p53 can only discriminate on the basis of expression levels and/or turnover. This is further complicated by the fact that recognition of the self-antigen results in deletion of the high-avidity T cells from the host repertoire during T cell development in the thymus by the process of central tolerance. Only T cells with low avidity for subdominant epitopes tend to escape this process. In fact, preclinical studies suggest “split tolerance” of p53, with high avidity CD8+ T cells deleted, while the CD4+ T cell repertoire is unaffected; this has been attributed to different requirements for antigen retention to induce tolerance via MHC class I versus MHC class II molecules. Another example of a TAA is telomerase reverse transcriptase (TERT), which is part of the telomerase complex that functions to maintain telomere length after cell division. This is an attractive target, because reactivation of TERT provides cells with unlimited proliferative potential, so its expression is a critical step in maintaining tumor growth. Thus, it is unlikely that antigen-loss variants will be selected in the face of an anti-TERT response.

C. Alterations in Post-Translational Protein Modifications

Tumor-specific aberrations in post-translational modification of protein antigens can result in novel antigenic structures that can be presented on MHC molecules, and thus recognized by T cells. For example, increased protein phosphorylation due to alterations in protein kinase activity can lead to unique TAA determinants. Alterations in glycosylation patterns can also generate new T
cell epitopes, such as tumor-specific modifications to the MUC1 glycoprotein observed in many adenocarcinomas.26

D. Antigens Expressed in the Stroma

Tumor growth is supported by a network of stromal cells, including macrophages, fibroblasts, and vascular endothelium, which express antigens typically associated with the normal processes of wound healing, neo-angiogenesis, and embryogenesis. Immunological targeting of the stroma is therefore a potentially useful anti-tumor strategy, although, as is the case for all TAAs, responses could be limited by tolerance mechanisms and potentially confounded by auto-immune complications. In fact, animal models have shown that it is possible to limit tumor growth without associated toxicity by inducing T cells for antigens expressed in angiogenic endothelia, including vascular endothelial growth factor (VEGF) receptor-2,28,29 matrix metalloproteinase-2,30 fibroblast growth factor receptor-1,31 survivin,32 and endoglin.33 In humans, several CTL epitopes have been characterized from proteins overexpressed by tumor neo-vessels, including VEGR-2,34 VEGFR-1,35 kinase insert domain-containing receptor (KDR),36 and fibroblast activation protein.37 Stromal cells are also capable of acquiring and presenting TSAs or TAAs derived from neighboring tumor cells, thereby becoming susceptible to lysis by tumor-specific CTL. This “bystander” killing of stromal cells has been shown to prevent the support of tumor variants in the face of an antigen-specific immune response.38 It has therefore been argued that the most effective tumor antigens will be those that can indeed be acquired and presented by the stroma.39

III. DENDRITIC CELLS AS INITIATORS OF ANTI-TUMOR T CELL RESPONSES

The initiation of a T cell response requires antigen uptake by DCs in the peripheral tissues, with subsequent transport to the lymphoid organs, or uptake by DCs already resident in the lymphoid organs. The antigens are processed into peptides and loaded onto MHC class I or class II molecules on the cell surface for presentation to CD8+ or CD4+ T cells, respectively. DCs need to differentiate further to acquire the capacity to induce the clonal proliferation of antigen-specific T cells. The terms DC “maturation,” “activation,” and “licensing” have been loosely used to encompass the phenotypic changes associated with this shift to greater stimulatory function. In fact, the differentiation process is not linear, and there are many factors that can act alone or in combination to affect the function of DCs. It is therefore more pertinent to refer to DCs according to their effector function—that is, the type of T cell response they induce, although this terminology can be cumbersome. Therefore, in this review, “maturation” will be used to refer to any process that provides DCs with a high expression of MHC and co-stimulatory molecules required to drive the proliferation of T cells.40 In contrast, the term “activation” will be reserved for the more potent process that converts resting DCs into effector DCs capable of priming T cells to both proliferate and differentiate into cytokine-producing cells. This process is dependent upon the release of cytokines by the DC itself, such as the cytokines interleukin (IL)-12 and IL-23.41-43 Whereas maturation can be induced by exposure to pro-inflammatory cytokines released by innate immune cells at sites of tissue perturbation or infection, activation generally involves interaction via receptors expressed by DCs. Examples of these are receptors recognizing “danger” stimuli; molecular structures associated with dead or dying tissue (damage-associated molecular patterns or DAMPs),44 or molecular structures typical of infectious pathogens (pathogen-associated molecular patterns or PAMPs).45 The term “licensed” will be used to describe DCs that have received feedback from effector lymphocytes in the form of molecular interactions, and cytokines that integrate with danger signals to further enhance the stimulatory function of DCs, providing T cell responses with the capacity for cytokine production and long-lived immunologic memory, a process that is particularly relevant for the induction of CD8+ T cell responses. Importantly, in the absence of danger stimuli, the presentation of antigenic peptides by DCs can actually result in tolerance of the antigen, with specific T cells being deleted or rendered unresponsive,46-50 or the DCs can induce regulatory T cells (Treg) with suppressor activity.49,51-53 It is therefore the
function of DCs to interpret the environmental context in which antigen has been acquired and presented, and determine whether immunity or tolerance ensues. Given the lack of obvious infectious agents contributing to most cancers, triggering of anti-tumor T cell responses is reliant to a large extent on DCs sensing the danger associated with localized cell death in the disorganized tissue of the tumor bed. It is potentially the failure of DCs to see tumors as dangerous that accounts for the fact that host immune responses in cancer patients are often weak and ineffective. These issues will be discussed in more detail later in this review.

A. Dendritic Cell Subtypes

It is now clear that there is heterogeneity in the DC phenotype, with subsets differing in location, migratory pathways, and immunological function. Much of the characterization of DC subsets, particularly with regard to function, has been established in the mouse, although some general conclusions can be drawn from studies in both mouse and human. One major division in phenotype is into “plasmacytoid” DCs (pDCs) and “conventional” DCs. The pDC subset only acquires the characteristic dendritic form and antigen-presenting function after activation, and is a major producer of type I interferons (IFNs) following exposure to viral or microbial agents. Accumulating evidence points to a role for type I IFNs in the differentiation of CD4+ T-helper cells into effectors of a Th1 phenotype characterized by release of IFNγ, and in the generation and activity of CTLs. Type I IFNs also generally support in vivo proliferation and survival of T cells. Together, these observations suggest that activated pDCs can have a significant role in anti-tumor immunity. In fact, injection of cultured murine pDCs has been shown to elicit antigen-specific CD8+ T cell responses, and to provide protection from tumor challenge. It has also been reported that human pDCs loaded with tumor antigens can prime IFNγ-secreting melanoma-specific CTLs in vitro. However, there remains some doubt that pDCs can actually acquire and present antigens from tumors in vivo, specifically with regard to presentation via MHC class I molecules to CD8+ T cells, a process called “cross-presentation” (to be discussed in more detail later). If pDCs play a role in cross-presentation, it may be to orchestrate this function in conventional DCs through the provision of type I IFNs. Finally, it should be noted that in cancer patients, pDCs can be seen in solid tumors in an immature state, and may in fact be responsible for inducing IL-10-producing CD4+ CD25+ Treg that inhibit anti-tumor immunity. Thus, without therapeutic intervention to activate tumor-infiltrating pDCs, these cells may actually represent a barrier to effective immunity.

The classification of conventional DC into “migratory” and “lymphoid-tissue-resident” cells is another major division applicable in mouse and human systems. Migratory DCs perform a sentinel role as immature cells in the peripheral tissues, and respond to danger in the form of cytokines and DAMPs or PAMPs by migrating to the draining lymph node with antigens they have acquired from sites of tissue perturbation. There is also a level of constitutive migration of DCs from peripheral tissues to the lymphoid organs so that, even in the absence of danger, populations of DCs with migratory phenotype can be detected in the lymph nodes and spleen. On the other hand, lymphoid-resident DCs seed the lymphoid tissues directly as precursors released from the bone marrow. In the steady state, lymphoid-resident DCs represent approximately half of the DCs in the lymph nodes and the majority of DCs in the spleen. Lymphoid-resident DCs respond to antigens (and activation signals) that circulate to the lymphoid tissue via the lymph or blood. These cells are not totally devoid of migratory activity; in the spleen, immature cells are found in the marginal zone and red pulp, but migrate to the T cell areas after exposure to maturation stimuli.

Murine lymphoid-tissue-resident DCs can be divided according to surface expression of CD8α-chain homodimers, with the CD8α+ DC subset being major producers of IL-12p70 after exposure to activation stimuli. These cells therefore play a key role in driving Th1-type responses. Significantly, CD8α+ DCs also have a heightened capacity to acquire dead cells and soluble antigens for cross-presentation to CD8+ T cells, thereby driving CTL responses. This process is a major requirement for the generation of CTLs to perturbed tissue, and therefore implicates CD8α+ DCs as major players in anti-tumor
responses should tumor material reach the lymphoid tissues. This is underlined by the fact that successful anti-tumor responses can be generated in mice when antigen is specifically targeted to lymphoid-resident CD8α+ DCs, typically by fusing antigen to antibodies specific for cell-surface receptors expressed by these cells; examples of such antibodies are DEC205 (CD205),75 Clec9,76 and langerin (CD207, expressed by a subset of CD8α+ DCs).77 Unfortunately, the CD8α homodimer is not expressed in human DCs, so it is hard to distinguish a corresponding subset to target in cancer patients. Nevertheless, based on the expression of markers shared between mouse and human DCs, a blood-borne human counterpart (BDCA3+ Necl2+ Clec9A+) has been suggested.76-80 Interestingly, while murine CD8α+ DCs have some ability to present acquired antigens on MHC class II molecules to activate CD4+ T cells, their CD8α+ DC counterparts (which can be subdivided further into CD8α+CD4+ and CD8α+CD4− double-negative DCs) are much more adept at this function,74,75 again underlining the fact that a division of labor exists between the DC subsets that must be considered in therapy.

A network of DCs is found within the peripheral tissues, and these are the precursors to the migratory cells found in the lymphoid tissues. The best characterized are the migratory DCs of the skin. At least three different DC subtypes exist in murine skin, which can be characterized by the expression of CD11c, the c-type lectin langerin, and the integrin CD103.81 Langerhans cells (LCs) are langerin+CD103− and are found distributed in the outermost layer of the epidermis, while two subsets of DCs are located in the dermis: langerin+CD103+ and langerin+CD103−. Similar CD103+ DCs have also been found in other peripheral sites such as the lung and gut.81-83 Following exposure to skin disruption and danger, LCs and dermal DCs can migrate to the draining lymph nodes, but may migrate to distinct areas, with LCs migrating to the T cell areas of the inner paracortex, and langerin+ dermal DCs to the outer paracortex just below the B cell follicles.84 This division in migratory function may therefore have an impact on the type of immune response induced. In fact, it has been postulated that a division of labor exists between human LCs and dermal DCs, with LCs promoting cellular responses, and dermal DCs humoral responses.85 However, the individual contribution of each of the migratory subsets to the priming T cell responses to skin tumors is currently unclear. The initiation of anti-tumor responses by epicutaneous vaccination has been studied in langerin-diphtheria toxin (DT) receptor mice, in which langerin+ cells can be ablated by injection of DT, with migratory langerin+ cells shown to cross-present vaccine-derived antigens to CD8+ T cells in the draining lymph nodes, although langerin+ dermal DCs contribute to the immune response when antigen doses are high.86 In other models, it has been shown that it is the langerin+CD103+ dermal DC subset that is most capable of cross-presenting antigens, although all three migratory subsets present antigen to CD4+ T cells.87 Cancer therapies have been developed based on injection of autologous DCs loaded with tumor antigen ex vivo, with the intention that the injected cells migrate to the lymphoid tissue to stimulate tumor-specific T cell responses. For practical reasons, this work has focused on DC subtypes that can be obtained in sufficient numbers. Most protocols have relied on the differentiation of DCs from blood-borne monocytes in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4. In fact, these cells are representative of “inflammatory DCs,” which are not normally found in steady-state, but are produced as a response to inflammation.65 These cells are therefore not the type of DCs that would encounter vaccine antigens injected into a patient, nor are they likely to be representative of DCs critically involved in host responses to tumors. Given the different functional roles attributed to DC subsets, further work may yet uncover a DC subtype that is more amenable to this kind of therapy.

B. Indications that Dendritic Cells Present Tumor Antigens

An early indication that cells of hematological origin were involved in priming anti-tumor CTL responses was provided by studies in chimeric mice, where bone marrow from donors of different MHC haplotypes (H-2b or H-2d) was engrafted into irradiated F1 hosts (H-2d x H-2b); in this way it was possible to establish whether induced anti-tumor CTLs were restricted to MHC mol-
ecules expressed by bone marrow or the MHCs encoded by the tumor tissue. The finding that CTLs were restricted to MHC molecules of the bone marrow challenged the previously held view that tumors present to T cells directly, and implied transfer of tumor antigens to host APCs. Whether these cells were in fact DCs has been harder to establish. The fact that DCs can be found within tumors, and can present tumor antigens to T cells in vitro, provides some assurance that DCs are involved, but perhaps the most convincing data are from recently developed animal models in which DC populations can be ablated in vivo, thereby allowing assessment of the role of these cells in disease situations.

1. Tumor-Infiltrating Dendritic Cells

It has been shown that DCs infiltrate tumor tissues in animal models of cancer, and tumor-infiltrating DCs (TIDCs) can be identified in clinical samples from cancer patients. However, purified TIDCs in mice and humans have been shown to be mostly of an immature phenotype, with low expression of co-stimulatory molecules, and incapable of stimulating naive tumor-specific CD8+ T cells in vitro. In fact, the tumor and its associated stroma have been shown to create an immunosuppressive environment with a profound effect on DC function. Altered myelopoiesis leads to the accumulation of undifferentiated myeloid cells in the bone marrow, blood, lymphoid organs and tumor tissue. These so-called myeloid-derived suppressor cells (MDSCs) are known to suppress T cell function through mechanisms involving the expression of L-arginase and/or nitric oxide synthase or the secretion of transforming growth factor-beta (TGF-β). Significantly, the tumor and its associated stroma also have profound local and systemic effects on DC function through the provision of cytokines such as IL-10, TGF-β, IL-6, VEGF, and M-CSF. Thus, a cycle of suppression ensues, which ultimately impairs the differentiation, maturation, and function of DCs in tumor-bearing hosts. Interestingly, direct intra-tumoral injection of activation stimuli such as PAMP structures that trigger Toll-like receptors (TLRs) on DCs, can induce tumor rejection in some murine tumor models. However, in some cases, the TIDCs remain refractory to this form of activation perhaps reflecting a higher level of immunosuppression. Similarly, TIDCs isolated from clinical samples often exhibit a “semi-mature” phenotype, with function only partially restored by stimulation with TLR agonists in vitro. Unleashing the full capacity of TIDCs to stimulate naive T cells may therefore require breaking the suppressive deadlock created by the tumor environment, as well as providing activatory stimuli for TIDCs. In support of this notion, TIDC paralysis in mice was reverted in one in vivo model by providing the TLR9 agonist CpG together with an anti-IL-10 receptor antibody to limit suppression, which lead to tumor rejection. The combined administration of multiple stimuli with synergistic impact on DC activation may overcome some suppressive qualities of the TIDCs themselves. For example, the combined activity of TLR3 and CD40 agonists decreased the L-arginase activity of TIDCs, and enhanced their production of type I IFNs and IL-12p70, resulting in effective T cell immunity in a model of ovarian carcinoma. This again underlines the fact that failure to induce effective anti-tumor responses may not simply be failure of DCs to recognize evidence of neoplasia as a dangerous condition worthy of response, but that an active process of suppression may be orchestrated by the tumor. It can perhaps be argued that if DCs do in fact control immunity to tumors, then the degree of DC infiltration of tumor tissue may hold some prognostic value. In fact, increased levels of TIDCs have been shown to be a positive prognostic factor in some cancers. However, other studies have failed to demonstrate a correlation between DC infiltration and survival, or even demonstrated a negative correlation between DC infiltration and tumor-free survival. In practice, prognosis is likely to reflect other important parameters of DC biology such as their ability to take up and present tumor antigen, their activation status, and the quality of responses they induce. For example, the frequency of TIDCs with a mature phenotype was associated with better survival in a cohort of patients with advanced melanoma. In another example, infiltration of human breast cancer tissue with mature DCs was shown to be unfavorable, which was explained by the ability of these DCs to drive CD4+ T cells that secrete IL-13, which facilitated tumor growth rather than inhibition.
2. Dendritic Cell Ablation Models

The development of transgenic mouse models lacking DCs has made it possible to specifically evaluate the role of tumor antigen presentation by DCs. In one model, a gene encoding the high-affinity human DT receptor (DTR) has been placed under control of the promoter for CD11c, a general “DC marker” in mice, enabling the depletion of all DCs by administration of DT. Using this model, the cross-presentation of tumor antigen from subcutaneously injected apoptotic/dying tumor cells was shown to be negated in the absence of CD11c+ cells. Similarly, the absence of CD11c+ cells negated tumor rejection induced by an agonistic anti-CD137 antibody. However, DT administration in mice was also shown to deplete marginal zone and metallophilic macrophages from the spleen and their sinusoidal counterparts in the lymph nodes, therefore making it difficult to completely attribute the observed effect to DCs. In addition, CD11c-DTR (DT-resistant) mice do not tolerate repeated injections of DT, possibly due to the off-target effects on other non-DC-cell populations, making it difficult to examine the role of DCs in priming anti-tumor T cell responses over a prolonged period of time.

In another study, mice deficient in the transcription factor Batf3, which lack lymphoid-resident CD8α+ DCs, were used. Interestingly, these mice were unable to control an otherwise immunogenic tumor due to inefficient priming of tumor-specific CD8+ T cells, while priming of CD4+ T cells was normal. This argues that CD8α+ DCs play a crucial role in cross-presentation of tumor antigens to CD8+ T cells, but a lesser role in the presentation of tumor antigens to CD4+ T cells. However, Batf3 deficiency also results in a lack of the CD103+ peripheral DCs in skin, and lungs, so these data do not conclusively rule out the possibility that dermal CD103+ DCs are also involved in the priming of tumor-specific CD8+ T cells, either directly presenting antigen or delivering antigen from subcutaneous tumors to the lymph nodes. While the authors showed that application of fluorescein-5-isothiocyanate and an irritant to the skin induced equivalent migration of fluorescent DCs to the lymph nodes regardless of Batf3 deficiency, there is some suggestion that dermal CD103+ DCs participate in an early wave of migration, which was not specifically tested.

The overall conclusion from the studies presented in this section is that DCs have the capacity to induce T cell responses to tumor antigens, but that in cancer patients this function is not optimal, and may in fact be actively suppressed by the tumor itself. Over the following sections we highlight some of the key processes that DCs would have to use to elicit T cell-mediated immunity to tumor tissue, discuss where these processes may be subverted by the tumor, and suggest some strategies to potentially overcome these barriers.

IV. UPTAKE OF TUMOR ANTIGENS BY DENDRITIC CELLS

A number of mechanisms have been described by which DCs can potentially acquire antigens from tumor cells. Perhaps the most relevant is uptake of dead or dying cells, which we will discuss in more detail below. However, a significant contribution from other forms of uptake cannot be discounted. These include uptake of free antigens, antigens chaperoned by heat shock proteins (HSPs) or immunoglobulins, uptake of antigen-bearing exosomes secreted by the tumor, or “nibbling” of plasma membrane and cytoplasm from tumor cells. Some processes may actually be independent of the uptake of whole antigens, such as direct transfer of peptide-MHC complexes from tumor to DCs or transfer of peptides through gap junctions.

A. Uptake of Dead or Dying Cells

Cell death is a common feature of tumors induced by triggers such as hypoxia, shortage of nutrients, or inefficient supply of growth factors, and can be induced by therapies such as radiotherapy or chemotherapy. The uptake of dying tumor cells by DCs is therefore likely to constitute an important source of tumor antigens. Recognition of apoptotic cells and phagocytosis can actually occur before membrane permeabilization and lysis of dying cells. Phagocytes must therefore be capable of recognizing early modifications to the cell membrane as “eat me” signals. The best characterized is a change in membrane asymmetry that results
in exposure of phosphatidylserine on the outside of the cell. Some proteins normally located on the inside of the cell, such as the endoplasmic reticulum (ER) protein calreticulin, also become exposed on the cell surface, and similarly serve as recognition signals. Other changes include modifications of existing surface molecules by oxidation (e.g., phospholipids) and alterations in sugar chains. These surface changes can permit interaction directly with receptors on phagocytes, or through bridge molecules (opsonins) that enhance the uptake of the dying cells by providing additional binding sites for receptor-mediated uptake. For example, phosphatidylserine can bind to Gas6 (growth arrest–specific gene 6) or protein S, and in turn be recognized by Mer, a member of the tyrosine kinase family of receptors on the phagocyte. Another bridging molecule is milk fat globulin–E8 (MFG-E8), which when bound to phosphatidylserine permits recognition via the vitronectin receptor (also called integrin αvβ3). Uptake of apoptotic cells can also be enhanced by serum or phagocyte-derived opsonins such as members of the collectin family eg. C1q, MBL (mannose-binding lectin), surfactant protein–A and –D, and C-reactive protein, all of which can bind to the low-density lipoprotein–receptor related protein CD91 complexed with calreticulin. Other receptors involved in the uptake of apoptotic cells include CD36 in complex with the integrin αvβ3 or αvβ5, class A scavenger receptor (SR–A), lectin-like oxidized low-density lipoprotein particle receptor-1 (LOX–1), and macrosialin (CD68).

While DCs express most of the receptors described above, and have been shown to efficiently present dead cell–derived antigen on both MHC class I and II molecules, some of the receptors involved are differentially expressed between DC subsets. For example, SR–A was demonstrated to be predominantly expressed by a subset of splenic double-negative DCs in mice, whereas CD36 is preferentially expressed in CD8α+ DCs and dermal DCs, and not by CD8α– DCs or LCs. Although studies in mice have since questioned the role of CD36 in the uptake of dying cells by CD8α+ DCs in mice, the above examples emphasize the need to understand more about the use of different receptors by DC subsets, especially given the differences in stimulatory function now being attributed to different DC subsets.

B. Tolerance or Immunity: Responses to Dying Tumor Cells

Efficient clearance of dead cells is an important process to avoid auto-immunity to self-antigens. However, the uptake of dying cells can under some circumstances induce the activation of adaptive immune responses. The uptake of dying tumor cells can therefore potentially have quite opposite outcomes, with the activation status of the DC a key determinant in this process. In this context, uptake of tumors undergoing apoptotic versus necrotic cell death has been suggested to be an important parameter in determining DC activation status. Apoptosis on the one hand is a natural part of normal tissue homeostasis, and the uptake of apoptotic cells is generally believed to be associated with immunological tolerance. Immature DCs that have acquired apoptotic cells become refractory to other activation signals and display a tolerogenic phenotype characterized by low expression of MHC and the co–stimulatory molecules CD40, CD80, and CD86, in combination with reduced release of inflammatory cytokines. Also, the uptake of apoptotic tumor cells by mononuclear phagocytes was demonstrated to be accompanied with secretion of immunosuppressive mediators, in particular TGF–β and IL–10, and has been associated with stimulation of regulatory T cells. Necrosis, on the other hand, is characterized by the rupture of the cell membrane as a result of swelling of the cytoplasm (necrosis). This leads to the release of a number of pro–inflammatory mediators such as IL–1 and tumor necrosis factor (TNF)–α, HSPs, and high–mobility group box (HMGB)–1, all factors that favor DC activation and therefore provide an environment in which antitumor T cell responses can be activated. Nevertheless, the difference in immunological outcome attributed to apoptotic versus necrotic cell death may be less clear-cut. A study in which mice were immunized with tumor cells treated with a variety of apoptosis–inducing drugs demonstrated that apoptotic cells could actually be immunogenic following some treatments. In particular, the anthracyclines and platinum–based
compounds induced immunogenic cell death by inducing both the translocation of the “eat me” signal calreticulin to the cell membrane, and the release of HMGB1. Thus, calreticulin facilitated phagocytosis of the dead cells, while HMGB1 induced efficient processing and presentation of antigens from dying cells through interaction with TLR4 on DCs.

Tumors are likely to contain mixtures of cells undergoing apoptosis, necrosis, and autophagy, which may reflect the tumor’s inability to cope with ongoing stress. It is therefore possible that interactions between DCs and apoptotic tumor cells interfere with immunostimulatory signals released by tumor cells undergoing necrotic cell death. In this vein, it was shown that the interaction of phosphatidylserine with DCs, which is characteristic of exposure to apoptotic cells, can make DCs refractory to activation by lipopolysaccharides in vitro. Also, blocking the interaction between DCs and phosphatidylserine in vitro and in vivo using a dominant negative form of MFG-E8 was shown to enhance protective immunity of a GM-CSF-expressing cancer cell vaccine in mice. In a similar fashion, blocking Mer, the receptor mediating phosphatidylserine binding through the bridging molecules Gas6 or protein S, abolished the suppressive effect of apoptotic cells and enhanced diabetes in a mouse model.

Finally, while interaction of DCs with early apoptotic cells suppresses DC activation, cells that have progressed to late apoptosis start to resemble necrotic cells in their ability to release inflammatory mediators and induce DC activation. Blocking phosphatidylserine-mediated uptake could therefore allow early apoptotic cells to progress through to late-stage apoptosis and induce DC activation, and reagents that block uptake of early apoptotic cells could therefore be novel agents for enhancing anti-tumor T cell responses.

V. ANTIGEN PROCESSING AND PRESENTATION BY DENDRITIC CELLS

Once DCs have acquired tumor antigens, the next step is to process the antigen appropriately for presentation to T cells. The classical antigen MHC class II presentation pathway, briefly reviewed below, provides the capacity for acquired antigens to be processed and presented on MHC molecules to CD4+ T cells. Presentation of acquired antigens on MHC class I molecules to CD8+ T cells is more problematic, because the classical MHC class I presentation pathway has been regarded as a means to present only antigens expressed within the cell, rather than those acquired from an exogenous source. There is now accumulating evidence that DCs are endowed with a remarkable capacity for diverting acquired antigens into the MHC class I presentation pathway. This pathway, termed cross-presentation, is widely regarded to be of particular importance in generating CTL responses to tumor tissue. A summary of these different pathways, with a particular emphasis on cross-presentation, is provided below.

A. Classical Antigen Presentation Pathways

The structures of MHC class I and II molecules are different, particularly with regard to the types of peptides they can present. Surface-expressed MHC class I molecules are complexed with β2-microglobulin, and present antigenic peptides that are normally restricted to eight to 10 amino acids in length. This limitation in length is forced by the structure of the peptide-binding groove of the MHC chain, with deep but highly conserved pockets at each end of the groove interacting with the termini of the peptide through extensive hydrogen bonding. In contrast, MHC class II molecules are formed from a combination of α- and β-chains, with both chains contributing to a binding groove that is open at both ends, allowing for binding of peptides up to 15 to 24 amino acids in length. The nature of antigens to be presented by MHC molecules is also ultimately defined by the intracellular compartments in which the MHC molecule samples antigen, and therefore the route in which it reaches the cell surface.

1. Presentation on MHC Class I Molecules

All nucleated cells express MHC class I molecules, which generally present peptides of endogenous origin, and are thus collectively representative of
the protein repertoire in the cell at the time of presentation. This repertoire includes proteins undergoing normal turnover within the cytosol, defective ribosomal products, and endoplasmic reticulum-derived proteins that are transported back to the cytosol for degradation. The process is initiated by targeting of proteins to the proteasome in the cytosol, which degrades the protein into peptides 2 to 25 amino acids long with appropriate carboxy-terminal amino acids required for MHC class I binding. This is followed by active transport of the derived peptides into the ER by the transporter associated with antigen processing (TAP), where further trimming at the amino terminus may be mediated by peptidases such as the ER-associated aminopeptidase 1 (ERAP1) in the ER. A subset of these peptides will have structural qualities that allow insertion into the peptide-binding domain of a given MHC class I molecule. The MHC class I molecules themselves are synthesized in the ER, where they are assembled together with β2-microglobulin and stabilized by the loading of peptide. This loading procedure, involving peptides of 8 to 10 amino acids, requires the transmembrane protein tapasin, which bridges MHC class I molecules to TAP, and the combined activity of the chaperone calreticulin and the oxidoreductase Erp57. After successful peptide loading, MHC class I molecules are then transported to the cell surface via the Golgi cisternae to present their peptides to CD8+ T cells.

While this pathway renders tumor cells susceptible to immune attack by activated CTLs, direct presentation of tumor antigens by this pathway in DCs is unlikely unless the DC itself is neoplastic. Therefore, the activation of tumor-specific CD8+ T cells by DCs must take place by cross-presentation. It is also important to note a significant difference in antigen processing between DCs and the constitutive pathway found in most tissues, or their neoplastic derivatives, which may have consequences for anti-tumor immunity. DCs express an “immunoproteasome” in which three different catalytic domains are recruited that favor cleavage behind hydrophobic or basic residues; this form of cleavage promotes appropriate carboxy-termini for MHC class I binding. As a consequence, a slightly different spectrum of peptides is generated in DCs relative to the constitutive proteasomes in other tissues. However, the immunoproteasome can be induced in tissues under the influence of IFN-γ, suggesting that optimal alignment between CTL priming and targeting of tumor tissue requires that the tumor tissue is exposed to inflammation. The corollary of this is that some determinants expressed by tumor cells in the absence of inflammation may not be produced, or are destroyed, by immunoproteasomes operating in DCs. Thus peptides presented on MHC class I molecules by tumor tissue may not always be appropriate determinants for responses generated via DCs; this fact must be considered in the design of therapeutic vaccines for the treatment of cancer.

2. Presentation on MHC Class II Molecules

The classical route of presentation of acquired exogenous antigens involves recirculation via the phagoslysosomal compartment and loading of antigenic peptides onto MHC class II molecules. MHC class II molecules are also synthesized in the ER, but their original assembly does not include peptide loading. Instead, they are associated with an invariant chain (Ii), which binds to the peptide-binding groove, stabilizing the MHC class II molecule until it is loaded with antigenic peptide. The cytoplasmic tail of Ii has an endosomal sorting and retention signal that targets MHC class II molecules to late endosomal “MHC II compartments”. The same compartments are a rich source of exogenous antigens recruited via fusion with phagolysosomal vesicles. The Ii chain is degraded by proteolytic cleavage, leaving a component associated with the binding groove, referred to as MHC class II-associated invariant-chain peptide (CLIP). The chaperone HLA-DM then coordinates the exchange of CLIP for antigenic peptides. Whether the peptides are generated before or after binding to MHC class II molecules remains to be elucidated. Because of the open binding groove, longer peptides can be inserted, with amino acids that are not hidden within the binding groove potentially exposed to further processing.

MHC class II molecules can be transported to the cell surface while still associated with Ii or after peptide loading. It is possible that transient transport to the cell surface followed by rapid internalization represents an important
part of the MHC class II pathway. In immature DCs, MHC class II molecules on the surface are frequently turned over via a ubiquitination-dependent mechanism, resulting in high intracellular concentrations. After DC maturation, these intracellular MHC class II molecules are efficiently loaded with peptides and transported to the surface, promoting long-lasting presentation of antigenic peptides, thereby improving stimulation of CD4+ T cell responses.

B. Cross-Presentation

It has long been recognized that a process of diverting acquired exogenous antigens to be presented by MHC class I molecules must operate in at least some APCs to explain the induction of CTL to agents that do not infect APCs. As early as the 1970s, Bevan provided evidence of such a phenomenon by showing that CTLs could be primed against cells bearing congenic minor histocompatibility antigens in mice. Bevan coined the term “cross-priming” to describe the process he observed, and the term cross-presentation has since been adopted to describe this pathway of antigen presentation.

Different intracellular processing pathways for cross-presentation have been described, although the individual contribution of the different pathways to cross-presentation in vivo is still controversial and seems to depend on the antigen type, uptake mechanism and differential processing of the antigen. While there is accumulating evidence to support a role for cross-presentation by DC in de novo responses to tumors, others have shown that tumor cells that can access the lymphoid compartment can directly stimulate CD8+ T cells. Interestingly, the model tumor antigen used in the latter studies, lymphocytic choriomeningitis virus glycoprotein (LCMV-GP), is now known to be “resistant” to cross-presentation unless modified to be retained in the ER. This in turn serves to reduce most proteolytic activities, with the exception of cathepsin S (CatS), which specializes in the production of relatively long peptides and therefore may favor cross-presentation.

There is now some evidence of heterogeneity even within the CD8α+ DC population with respect to cross-presentation. One recent study exploited the requirement for cross-presentation with whole tumor antigens. We therefore give an overview of current models of cross-presentation below (summarized in Fig. 1), although it should be noted that much of this knowledge has been garnered outside of the cancer field.

1. Heterogeneity of Dendritic Cells and Cross-Presentation

Cross-presentation has been observed in a variety of APCs, although DCs have been shown to be the most efficient in vivo. The superior cross-presentation activity attributed to DCs relative to other cell-types, such as macrophages and neutrophils, has been attributed to the fact that antigens are generally internalized into phagosomes with an oxidative environment and a nearly neutral pH, which permits only low levels of proteolytic activity. The emphasis is therefore on retention of antigen for presentation purposes, rather than on the rapid degradation and elimination that occurs in other phagocytic cells. Thus, expression and recruitment of proteolytic enzymes to phagosomes of DCs is low, there is only limited acidification by the V-ATPase, and an active process alkalinization operates via activity of the NADPH oxidase NOX2.

In fact, as noted in an earlier section, there is heterogeneity in cross-presenting function among DC subsets, which is exemplified by the CD8α+ subset of splenic DCs in mice. The superior cross-presentation by CD8α+ DCs is not a function of increased Ag uptake but is more likely to be caused by differences in antigen processing. In this context, CD8α+ DCs have been shown to express higher levels of proteins of the MHC class I machinery than other splenic DC subsets, and their distribution and expression of Rac1 and 2 GTPases, which ultimately control the assembly of NOX2 subunits, lead to the pH being higher in phagosomes of CD8α+ DCs than in other DC subsets. This in turn serves to reduce most proteolytic activities, with the exception of cathepsin S (CatS), which specializes in the production of relatively long peptides and therefore may favor cross-presentation.

There is now some evidence of heterogeneity even within the CD8α+ DC population with respect to cross-presentation. One recent study exploited the requirement for cross-presented
antigens to be diverted into the cytosol to deplete cells with a heightened propensity for the cross-presentation process. This was accomplished by injecting mice with horse cytochrome c, which induces "suicide" in cells that acquire and divert this pro-apoptotic protein to the cytosol.\textsuperscript{220} While the CD8α+ DC population was significantly depleted by this treatment, a proportion of CD8α+ DCs were not affected by cytochrome c administration, suggesting that these remaining cells were incapable of cross-presentation. In a more recent study, Qiu et al. showed that among splenic CD8α+ DCs, the CD103+ langerin(CD207)+ subset was responsible for phagocytosis of apoptotic cells and tolerance of cell-associated antigens via cross-presentation.\textsuperscript{221} Langerin+ DCs may be particularly effective at screening the blood for self-antigens by virtue of their location in the marginal zone of the spleen.\textsuperscript{67,222} Under the appropriate conditions, these same cells may be responsible for stimulating immunity, as suggested by a study in which antigen was targeted to langerin+ cells with an anti-langerin antibody.\textsuperscript{77} Our own studies have shown that langerin+ CD8α+ DCs are primarily responsible for cross-presentation, and have the capacity to drive potent antigen-specific CTL responses if
combined with the appropriate activation stimuli such as TLR stimulation or concomitant activation of NKT cells to provide “licensing” signals (discussed later).\textsuperscript{223} The production of IL-12 in response to systemic activation stimuli is also dependent on langerin\textsuperscript{+} CD8\textsuperscript{a} DCs, with the ratio of IL-12p40 to bioactive IL-12p70 manufactured determined by the activation stimulus used.\textsuperscript{223} The langerin\textsuperscript{+} subset of CD8\textsuperscript{a} DCs is therefore crucially involved in priming and differentiation of responses to cross-presented antigen. The involvement of these cells in de novo responses to tumors has yet to be investigated, as does the possibility that specific targeting of these APC by vaccination will improve immunotherapy.

This is not to suggest that the other DC subsets of the murine spleen (or other lymphoid organs) are incapable of cross-presentation. Antigen that has been incorporated into immune complexes has been shown to be cross-presented by CD8\textsuperscript{–} DCs.\textsuperscript{224} We recently reported that antigen conjugated to a modified superantigen construct that targeted MHC class II molecules provided access to an undefined pathway of cross-presentation operating in all DC populations assessed, including the three major DC subtypes in spleen.\textsuperscript{225} However, the evidence of heterogeneity of DC function in the mouse, particularly with respect to cross-presentation, suggests similar diversity in humans, and suggests that more information on the function of human DC subsets would be informative.

2. Cytosolic Pathway of Cross-Presentation

Many studies of cross-presentation, including initial studies of the involvement of APCs in an antitumor context,\textsuperscript{226} have shown a requirement for cytosolic proteolysis and transporters associated with antigen processing (TAP1 and TAP2). The acquired exogenous antigens must therefore be diverted from phagosomes (or endosomes if uptake is specifically receptor mediated) into the cytosol for degradation and subsequent transport of peptide fragments into the ER, where peptide loading of MHC molecules takes place. However, the mode of exit of antigen from the phagosomes is still undefined. Some polypeptide sequences may be capable of simply “leaking” through the membranes.\textsuperscript{227,228} An example is a decameric polypeptide fragment of HIV-Tat, which can translocate itself across membranes in an energy-independent manner. By interacting with phosphate groups on either side of the membrane, it may also form a pore to aid translocation of other material.\textsuperscript{229} It is possible that many other peptides exhibit such a capacity. It is also possible that the phagolysosome actually ruptures, releasing its contents into the cytosol. It is known that lysosomal membrane stability is regulated by host proteins such as sphingosine, so it has been speculated that there may be cell-specific control over phagosomal membrane integrity that may aid in antigen transfer.\textsuperscript{230} Significantly, deliberately forcing phagosomal membrane perturbation can indeed promote cross-presentation. An example is listeriolysin, a major virulence factor of \textit{Listeria monocytogenes} that has been shown to perforate the phagosomal membrane and facilitate egression of the bacterium into the cytosol. Listeriolysin-aided delivery of antigen into the cytosol has been shown to enhance cytosolic delivery and thereby improve CTL induction by cross-presentation,\textsuperscript{231} a process that could be exploited in the design of antitumor vaccines.

3. ER-Phagosome Fusion Pathway of Cross-Presentation

Phagosomes in DCs and macrophages have been shown to contain ER-associated components of the MHC class I loading pathway,\textsuperscript{232-234} which is thought to result from a fusion event between phagosomes and the ER.\textsuperscript{235} This conveniently allows MHC class I loading directly in the fused vesicle, or “ergosome.”\textsuperscript{234} In fact, the likely route is circuitous, with antigen having to egress the ergosome to access the proteasome (which may in fact be membrane associated on the cytosolic face), and then re-enter the ergosome via TAP for MHC class I loading.\textsuperscript{232,233,236} A recognized process for retro-translocation of proteins from the ER into the cytosol that operates in most cells, called the ER-associated protein degradation (ERAD) pathway, may be responsible for depositing the antigen in the cytosol. The ERAD pathway is known to target misfolded proteins of the ER to the cytosol for ubiquitination and subsequent degradation. A protein complex called Sec61 provides the channel necessary for this transport
process. Significantly, it has been reported that cross-presentation is reduced when Sec61 is specifically inhibited by RNA interference\textsuperscript{237} or its function is blocked with \textit{Pseudomonas aeruginosa} exotoxin A.\textsuperscript{238} Interestingly, non-phagocytic cells can be rendered competent for cross-presentation by simply making them phagocytic through the expression of FcγIIA receptors, implying that DCs may have merely adapted common features of the ERAD pathway for the specialized process of cross-presentation.\textsuperscript{239} Further evidence for the existence of an ER-fusion pathway comes from studies showing that TAP can be specifically inhibited in early endosomes, which prevented cross-presentation.\textsuperscript{240} However, other authors have challenged the ER-phagosome fusion pathway model because they observed only a very low contribution of the ER to the phagosome membrane in their studies,\textsuperscript{241} meaning that the process would be inefficient. One possible explanation is that lipid bodies, storage organelles rich in cholesteryl esters or triglycerides that can participate in protein egress from the ER,\textsuperscript{242} may actually aid the transfer of the required ER-associated proteins directly to the phagosome.\textsuperscript{230} Intriguingly, it was recently observed that DCs deficient in the IFN-inducible, ER-resident GTPase, Irgm3, displayed markedly fewer lipid bodies than wild-type DCs, and failed to cross-present phagocytosed antigens efficiently, while presentation to CD4\textsuperscript{+} T cells was unaffected.\textsuperscript{243}

4. Endosome-to-ER Pathway of Cross-Presentation

Whereas particulate antigens acquired by phagocytosis may have access to ergosomes described above, soluble proteins acquired by macropinocytosis may actually directly enter the lumen of the ER.\textsuperscript{244} It was demonstrated that conventional presentation of endogenous antigens on surface MHC class I molecules could be restored in β2-microglobulin-deficient cells by the addition of soluble β2-microglobulin, implying that the intact β2-microglobulin protein gained access to MHC class I molecules in the ER. In addition, both conventional MHC class I presentation and cross-presentation could be inhibited by the addition of soluble US6 protein, an inhibitor of TAP function, again suggesting that intact soluble protein can gain access to the ER.\textsuperscript{244} Antigens that enter the ER may then be translocated into the cytosol by the ERAD pathway, and their derived peptides transported back into the ER for loading onto MHC class I molecules.

5. Tap-Independent Cross-Presentation

Some cross-presentation can be observed in the absence of classical MHC class I pathway-associated components such as TAP, or the cytosolic proteasome.\textsuperscript{245-249} Cross-presentation by this TAP-independent pathway is not significantly inhibited by brefeldin A, indicating that loading of MHC class I molecules occurs after MHC class I molecules are exported via Golgi cisternae.\textsuperscript{249} The pathway is inhibited by chloroquininone, suggesting a dependence on lysosomal processing. Thus, this pathway is likely to involve peptide exchange in recycling endosomes. It is well known that both MHC class I and class II molecules are recycled between endosomal compartments and the cell surface.\textsuperscript{248,250,251} Because internalized MHC class I molecules already harbor peptides,\textsuperscript{252} there must be an exchange process for newly generated peptides, which may be favored by the environment of specific recycling endosomes. In this context, the pH of the endosomes may play significant a role, with pH 5.0 being optimal for the release of existing peptides and exchange for high-affinity peptides, but a lower pH being unfavorable because it permits β2m to dissociate from the MHC class I heavy chain.\textsuperscript{253} In vivo evidence for a TAP-independent cross-presentation pathway comes from a study in which proliferation of ovalbumin (OVA)-specific T cells was induced in TAP-deficient mice after the administration of OVA protein incorporated into polylactide polyglycolide copolymer microspheres.\textsuperscript{254} Interestingly, the response was considerably reduced in animals deficient in both TAP and CatS, implying a key role for CatS in this pathway. A recent examination of the internalization of MHC class I in DCs demonstrated a tyrosine-based endocytic trafficking motif that is required for the constitutive internalization into early endosomes, and then deep into lysosomal peptide-loading compartments, which if deleted, impaired cross-presentation.\textsuperscript{255,256}

The internalization of MHC class II molecules may also feed into compartments that favor
cross-presentation. A recent study highlighted a clathrin-independent pathway of MHC class II internalization that involved Arf6⁺, Rab65⁺, EHD1⁺ endosomal tubules. Interestingly, internalized MHC class I molecules can also recirculate through Arf6⁺ endosomes, suggesting that reloading of both classes of MHC molecule may be possible in these compartments. In this context, antigen specifically targeted to MHC class II molecules in vivo by conjugation to a modified superantigen construct was shown to be very efficiently cross-presented by DCs, perhaps as a result of being internalized into “reloading” compartments.

6. Gap Junctions and Cross-Presentation

Gap junctions are small pores formed between cells for the intercellular transport of nutrients and other small molecules. It has been shown that pores formed by oligomerization of connexin 43 (Cx43) can allow peptides of up to 10 amino acids long to pass between cells. By establishing gap-junctional contact with local cells, APCs may be able to acquire peptides for presentation on MHC class I. Because peptides are rapidly degraded within the cytosol, it is likely that the spread of peptides through gap junctions will be rather limited, perhaps no more than one cell distant from the antigen source. Some APCs, including monocytes and DCs, up-regulate Cx43 after receiving activation signals, and cultured human DCs were shown to increase the expression of Cx43 after exposure to TNF-α and melanoma lysates, which facilitated cross-presentation to melanoma-specific CTL clones. Also, because gap junctions are operational until apoptotic cells remodel to form apoptotic bodies, transfer can also theoretically occur between apoptotic tumor cells and DCs. Finally, the network of stromal cells that support tumor growth may acquire and cross-present antigenic peptides from neighboring tumor cells, and thus become targets for CTLs themselves.

7. Prolonged Cross-Presentation

While the classical pathway of MHC class I presentation provides an up-to-date “inventory” of the internal content of the cell, DCs engaged in cross-presentation may be called upon to engulf antigens in peripheral tissues and then present them in the T cell zones of the lymphoid organs without encountering a further source of the same antigens. Because this migration time may take 24 to 48 h, a capacity for long-term storage of acquired antigens would be desirable to sustain high levels of peptide MHC complexes when DCs eventually engage antigen-specific T cells. In fact, it has been shown that cross-presented peptides are rapidly replenished at the cell surface following elution of peptide from MHC class I molecules on the surface of DCs. A lysosome-like organelle distinct from either the MHC class II compartments or the early endosomal compartments involved in acute cross-presentation was characterized as the intracellular depot. Peptide display lasting as long as 14 d was achieved when the exogenous antigen was conjugated to a TLR ligand or IgG, indicating that endocytic uptake combined with triggering of activation was required for optimal depot formation.

VI. ‘DANGER’ AND DENDRITIC CELLS IN THE TUMOR CONTEXT

The induction of an effective anti-tumor T cell response requires antigen uptake and processing by DCs in the context of environmental signals that suggest danger to the host. The identification of pattern recognition receptors (PRRs) that recognize conserved PAMPs helped to explain how the immune system is alerted to regions of infection, with the TLR family of PRRs playing a significant role. Since then, it has become clear that cellular stress alone can induce the expression of endogenous ligands that act as “danger” signals. These DAMPs are induced or released by injured cells, and while there is some crossover in receptor use to detect PAMPs and DAMPs, the identification of specialized DAMP receptors is now a major area of research. A number of DAMPs have now been identified that are released by dead or dying tumor cells, and therefore potentially play a significant role in anti-tumor immunity.

A. HSPs

HSPs comprise a group of molecular chaperones involved in protein folding, maintaining protein
conformation, and preventing unwanted protein aggregation. Significantly, their expression is up-regulated in response to heat, oxidative stress, glucose starvation, or other stress factors, suggesting they could be recognized as danger signals. In this context, mitochondrial HSP60 and cytosolic HSP70 and HSP90 have been shown to be translocated to the cell surface in response to stress, and are therefore available for direct interaction with DCs. It was recently demonstrated that HSP90 expression was increased when human myeloma cells were treated with the 26S proteasome inhibitor bortezomib, and that these cells showed greater immunogenicity compared with gamma-irradiation or dexamethasone treatment, which caused a similar level of apoptosis. This increased immunogenicity was dependent on direct contact between DCs and the treated tumor cells, and was abrogated by blocking HSP90, demonstrating a role for HSP90 as a DAMP with activatory properties. Immunostimulatory activity of tumor cells enriched in HSPs by periods of hyperthermia has also been observed in some models. HSPs can induce DC activation in a TLR-dependent manner, suggesting that HSPs encode an appropriate TLR ligand, or carry one, or simply that HSP preparations contain contaminants. Nevertheless, cell surface expression of HSP-70 and gp96 in eukaryotic cells has been shown to induce DC maturation and IL-12 secretion in a MyD88-dependent manner, ruling out an effect of contaminating bacterial endotoxins that may have confounded results in experiments with synthesized HSPs.

It is possible that HSPs can serve dual roles as both antigen couriers and danger signals. Mice immunized with HSP70, HSP90, or HSP96 purified from tumor tissue were protected against tumor challenge with the same tumors, which has been attributed to the capacity of HSPs to bind tumor peptides and mediate their cross-presentation in vivo. Furthermore, APCs pulsed with HSP96 purified from human melanoma cell lines were recognized by CTLs specific for the melanoma antigen MART-1 and not by CTLs specific for the colon carcinoma antigen EP-CAM1; the opposite was true for APCs pulsed with colon carcinoma-derived HSP96. This suggests that HSP96 provides antigens for cross-presentation that are specific for the cell line from which they were purified. The uptake of HSP/peptide complexes is via scavenger receptors on the DC, including CD91, which was shown to mediate uptake of HSP96, HSP90, HSP70, and calreticulin. However, the capacity for HSPs to select and bind peptides, and their role in cross-presentation, is controversial, with some arguing that HSPs are very weak immunogens compared with other antigen preparations such as a simple boiling procedure.

B. HMGB1

Passive release of HMGB1 from necrotic cells was demonstrated to induce TNF-α production by macrophages, which was not observed when HMGB1–deficient cells were used, suggesting that HMGB1 serves as an endogenous danger signal. Normally located in the nucleus, HMGB1 is a chromatin-binding protein involved in the assembly of nucleoprotein complexes, and thereby indirectly involved in modulating the transcriptional activity of genes such as the steroid hormone receptors, NF-κB, p53, RAG1 recombinase, and homeobox-containing proteins. The release of HMGB1 into the cytoplasm in dying tumor cells, and then subsequent extracellular release, has been shown to trigger activation of DCs. Three receptors on DCs are involved: receptor for advanced glycation end products (RAGE), TLR2, and TLR4. Triggering of TLR4 on DCs by HMGB1 has also been reported to inhibit fusion of phagosomes with lysosomes, thereby preventing the rapid degradation of antigen and favoring antigen presentation. Furthermore, immunization with tumor cells that had been induced to undergo an immunogenic cell death by certain anticancer chemotherapies protected wild-type mice against a subsequent tumor challenge but failed to do so in TLR4–deficient animals. The lack of protection in TLR4–deficient animals was attributed to a lack of HMGB1 triggering of TLR4 on DCs, demonstrating a unique role of HMGB1 as a danger signal.

C. Uric Acid

A low molecular fraction purified from the supernatant of dying cells that could activate DCs was identified as uric acid, an end product of the cell-
lular catabolism of purines.\textsuperscript{288} Uric acid is present at high concentrations in the cytosol of healthy cells, but when it is released from dying cells and comes into contact with high levels of free sodium in the extracellular environment, it forms monosodium urate crystals. Monosodium urate is considered to be the biologically active structure responsible for the danger signal provided to DCs, which is mediated through engagement of the caspase-1-activating NALP3 inflammasome, resulting in the production of active IL-1\(\beta\) and IL-18.\textsuperscript{289} The significance of uric acid as a danger signal was highlighted by studies in which the elimination of uric acid reduced the generation of CTLs to an antigen in transplanted syngeneic cells, and also reduced the proliferation of autoreactive T cells in a transgenic diabetes model. In contrast, uric acid depletion did not reduce the stimulation of T cells to mature DCs or when endogenous APCs were activated with anti-CD40 antibody.\textsuperscript{290} Significantly, uric acid levels are elevated in tumors undergoing immune rejection, and the inhibition of uric acid production by systemic administration of allopurinol, or the removal of uric acid by administration of uricase, delays tumor immune rejection. In contrast, subcutaneous administration of crystalline uric acid was shown to enhance the tumor rejection process.\textsuperscript{291}

### D. Extracellular ATP

While the nucleotide ATP is primarily known for its role as an energy source, it can also serve as a DAMP signal.\textsuperscript{292,293} Like uric acid, ATP is found in high concentrations in healthy cells, but in low concentrations in the extracellular environment, so that cell injury results in its rapid release.\textsuperscript{294-296} DCs exposed to extracellular ATP up-regulate co-stimulatory molecules, secrete IL-12, and exhibit an improved stimulatory capacity for T cells.\textsuperscript{297,298} This interaction involves P2X7 purinergic receptors on DCs, and triggers the secretion of IL-1\(\beta\)\textsuperscript{299,300} through activation of the NOD-like receptor family pyrin domain containing-3 protein (NLRP3)-dependent caspase-1 activation complex (otherwise known as the “inflammasome”).\textsuperscript{301,302} Interestingly, the release of ATP from tumor cells treated with the chemotherapeutic reagent oxaliplatin (belonging to the anthracycline family) was recently shown to enhance anti-tumor T cell priming by inflammasome-dependent IL-1\(\beta\) release.\textsuperscript{303} This pathway was also demonstrated to be of clinical relevance, because a loss-of-function polymorphism in P2RX, that lowers the affinity of for ATP was shown to have a negative prognostic impact on disease-free survival in breast cancer patients treated with anthracycline.\textsuperscript{303}

### E. Triggering of CLRs

The C-type lectin receptor (CLR) family is a diverse group of receptors with a C-type lectin domain typically involved in binding of carbohydrate structures in a calcium-dependent manner. Many myeloid cells, including DCs, express carbohydrate-binding CLRs as well as “non-classical” C-type lectins that lack the residues involved in calcium binding. Recently, it has become clear that some CLRs are involved in eliciting immune responses to dying cells through their recognition of DAMP signals. For example, Mincle (also called C-type lectin [Clec]-4e or Clecfs9) recognizes SAP130, which is a component of the U2 small nuclear ribonucleoprotein-associated protein complex released from dying cells.\textsuperscript{304} In this context, the Mincle receptor itself does not initiate signaling but is dependent on interaction with the Fc\(\gamma\) receptor for downstream tyrosine kinase activation and APC activation.

Another receptor, Clec9A, has been shown to be involved in the recognition of antigenic material from dead cells, although the specific ligand is currently unknown (apart from being protease susceptible but resistant to glycosidase and nuclease treatment).\textsuperscript{305} Despite being regarded as an endocytic receptor, Clec9A does not appear to be involved in the phagocytosis of dead cells. Instead, DCs deficient in Clec9A are unable to cross-present antigen once the dead material has been acquired. It has therefore been proposed that Clec9A is involved in diverting antigen away from lysosomal compartments to allow more antigen to be cross-presented.\textsuperscript{305} Interestingly, targeting of antigen to Clec9A with a specific antibody did not result in obvious DC activation, yet did result in induction of an immune response. Triggering of Clec9A therefore may not function as a potent danger signal per se, despite the existence of an intracellular immunoglobulin family tyrosine-based activation motif (ITAM), but may simply
increase cross-presentation to a point at which a mild activation trigger is all that is required.\textsuperscript{76}

As the previous example attests, some CLRs may be able to discriminate dead or dying tissue, or even neoplastic tissue, from normal, but the outcome of these interactions in terms of tolerance or immunity is not only determined by the signaling qualities of the CLR itself, but may also depend on the integration with signals from other PRRs. For example, the triggering of DC-SIGN can integrate with nuclear factor-κB signaling induced by TLR-3, TLR-4, or TLR-5 to modify DC activation,\textsuperscript{306-308} and was demonstrated to suppress TLR-induced DC activation induced by \textit{Mycobacterium tuberculosis}.\textsuperscript{307} Others, like Dectin-1, Dectin-2, and Mincl can induce gene expression and cytokine production by DCs independent of other PRRs, either through their own ITAM or by recruiting ITAM-containing signaling molecules such FcγR.\textsuperscript{304,309-311} A third group, including Clec12A and DCIR, contain immunoreceptor tyrosine-based inhibition motifs (ITIMs) in their cytoplasmic domains, and have been reported to suppress cytokine responses mediated by other PRRs.\textsuperscript{312-316}

It is perhaps useful at this point to note some interactions provided by CLRs that may be of immunotherapeutic use in the tumor setting, with the caveat that the net outcome of CLR recognition may depend on additional signals for effective immunity. Thus, some CLRs expressed by DCs can potentially participate in anti-tumor responses by detecting tumor-specific glycan changes. For example, MGL/CD301 detects glycan changes on MUC1 in colon carcinoma,\textsuperscript{317} and DC-SIGN recognizes tumor-specific glycosylation of carcinomaembryonic antigen on colorectal cancer cells.\textsuperscript{318} In mice, DEC205 has been shown to be involved in the uptake of dying cells, although currently the function and ligand specificity of DEC205 is unknown.\textsuperscript{319} Interestingly, in the mouse, DEC205 is preferentially expressed by CD8α+ DCs, which are predisposed toward cross-presentation, so that targeting antigen to this receptor results in efficient uptake and cross-presentation, although some form of adjuvant is required for CTL induction.\textsuperscript{75,320} This tactic of exploiting the unique distribution of CLRs between the different DC subsets may be of significant therapeutic value, because it permits targeting DCs with specific functional attributes. Thus, in addition to DEC205, the CD8α+ DC subset can be targeted via the CLRs CLEC9A,\textsuperscript{76,80} CLEC12A,\textsuperscript{321} and langerin\textsuperscript{77} (although this targeting will not be entirely exclusive; CLEC9A and CLEC12A are expressed to a lesser extent on pDCs, and langerin is expressed by LCs, CD103+ dermal DCs, and possibly other CD103+ peripheral DCs). On the other hand, DC-SIGN,\textsuperscript{322} Dectin-1 and -2,\textsuperscript{323,324} and DCIR\textsuperscript{275,325} can be targeted on CD8α+ DCs. Targeting to DCIR2 has previously been shown to favor CD4+ T cell responses.\textsuperscript{75} It remains to be seen whether such specific targeting of DC subsets via their CLRs can be achieved in humans.

\section*{F. Triggering of Antibody Receptors}

Antibody-mediated uptake of antigen has been demonstrated to enhance T cell priming in response to free antigen and apoptotic tumor cells compared with non-opsonized antigen.\textsuperscript{116,224,326-330} While generally not characterized as a danger signal, antibody-opsonized antigens are detected by Fcγ receptors FcγR that can influence DC activation status.\textsuperscript{326,331} In fact, the outcome of FcγR receptor ligation depends on the balance between stimulatory and inhibitory Fcγ receptors, with some antibody isotypes having stronger binding preference for one type over the other.\textsuperscript{331-334} For example, mouse IgG1 antibodies bind the inhibitory receptor FcγRIIB with higher affinity than the activating receptor FcγRIII, while IgG2a antibodies bind the activating receptor FcγRIV with higher affinity than FcγRIIB.\textsuperscript{333,335} It follows that enhanced DC activation (and cross-presentation) can be achieved by blocking inhibitory FcγRs\textsuperscript{336,337} or by choosing antibody isotypes that bind preferentially to activatory FcγRs when using opsonized tumor material therapeutically, or it may be possible to engineer the Fc portion so that they preferentially bind these receptors.

Uptake of antibody-opsonized tumor cells via FcγR receptors allocates antigen to an ERAD-dependent pathway of cross-presentation.\textsuperscript{239} In addition, DCs possess the ability to direct antibody-opsonized antigen into an intracellular compartment from which antigen can be slowly released, effectively prolonging the duration of cross-presentation of tumor antigens.\textsuperscript{263} The importance of FcγR-mediated uptake of tumor antigen is highlighted by studies with the com-
monly used anticancer monoclonal antibodies Rituximab (anti-CD20) and Trastuzumab (anti-Her2/ErbB2). While these antibodies mediate tumor cell killing by antibody-dependent cellular cytotoxicity or complement-mediated killing, it has become clear that an important part of the clinical responses they induce is mediated by enhanced priming of anti-tumor T cell responses due to enhanced cross-priming and DC activation induced by FcγR-mediated uptake of tumor antigen.338

VII. LICENSING OF DENDRITIC CELLS

Although many cancer cells and their associated stroma can trigger DAMP signaling in DCs, this may still be insufficient to provide full effector function in responding T cells. Importantly, T cells themselves can also modify the function of DCs, so that it is likely that additive, or even synergistic, integration of these signals with DAMP signaling ultimately determines whether a quality anti-tumor T cell response is induced.

A. Licensing by CD4+ T Cells

One significant interaction between T cells and DCs is via CD40, which is expressed constitutively on DCs (albeit at low levels until a maturation stimulus is provided), and CD40L (also known as CD154), which is expressed transiently by activated CD4+ T cells. Triggering of CD40 is known to integrate effectively with PRR signaling to enhance DC function, including increased expression of co-stimulatory molecules and release of pro-inflammatory cytokines, such that DCs gain a significantly enhanced capacity to stimulate CD8+ T cell responses. Thus, CD4+ T cells indirectly “help” CD8+ T cells by cognate interaction with DCs, a process that has been termed DC “licensing.”339-341 It is possible that CD40 signaling specifically acts on components that favor cross-presentation, rather than presentation via the classical MHC class I pathway, so that the heightened stimulatory capacity is reserved for CD8+ T cells specific for acquired antigens.342 Primary responses induced in the absence of DC licensing tend to be of a smaller magnitude, although the most profound difference is a reduced ability to evolve an effective memory population.343

Noninfectious agents have demonstrated a greater need for cognate CD4+ T cell help to drive CD8+ T cell responses than infectious agents, and CD8+ T cell responses to some infectious agents are completely independent of CD4+ T cell help. There is increasing evidence to suggest that it is the quality of pattern recognition by DCs that determines this requirement. Two observations have helped explain this variation in helper dependence. First, CD40L can be induced in DCs by some, but not all, TLR ligands,344 and second, CD40 can be expressed on CD8+ T cells after activation.345 Thus, pathogens that provide appropriate TLR stimuli to provoke CD40L expression on DCs can trigger improved CD8+ T cell function via direct interaction with CD40 on the CD8+ T cell, obviating the requirement for an intermediary CD4+ T cell.344

It is possible that failure to receive T cell help is one of the factors holding anti-tumor CD8+ T cell responses in check. However, through understanding the processes of T cell help invoked in infection, it may be possible to design therapies that trigger help, or perhaps eliminate the need for it. An obvious candidate is agonistic anti-CD40, which should trigger licensing of DC as well as directly stimulating CD40 on CD8+ T cells. In mice, failure to raise an effective CTL response against established tumors expressing the human adenovirus E1A antigen was overcome by systemic or intratumoral administration of anti-CD40 antibodies, which acted on host cells.346,347 Administration of anti-CD40 antibody induced effective anti-tumor CTLs from CD8+ T cells that would otherwise have been deleted in non-sporadic models of tumorigenesis with SV40 large T antigen expressed under the rat insulin promoter or the alpha-amylase promoter,348 reflecting improved activation status of APCs in vivo.

Understanding why stimulation through some TLRs, such as TLR3 and TLR9, contributes to helper independence while others, including TLR2, TLR4, and TLR7, do not344 may also inform better therapies. It has been suggested that TLR-induced release of type I IFN, which can be induced by both TLR3 and TLR9 agonists, may be involved.350 In fact, type I IFN has already been shown to stimulate CD4+ T cell-independent priming of CTL responses.351 Finally, a system-
atic analysis of pattern recognition focusing on ligands that provoke CD40L expression by DCs may uncover useful agents for improving tumor immunotherapy by reducing the requirement for T cell help.

B. Licensing by NKT Cells

Sub-populations of T cells with invariant or semi-invariant antigen receptors, such as those restricted by CD1 molecules, can potentially provide a potent source of stimulatory signals for DCs by virtue of their high frequency,\textsuperscript{352-355} and also because many of these populations exhibit a phenotype typical of activated cells that includes expression of CD40L. Significant among these populations are invariant NKT cells expressing an invariant TCR \(\alpha\)-chain encoded by \(\text{V}z14-J\alpha18\) gene segments in mice or \(\text{V}z24-J\alpha18\) in humans,\textsuperscript{356} and a restricted V\(\beta\) repertoire.\textsuperscript{356-358} These cells are found at high frequency in the spleen, bone marrow, thymus, and liver, and respond to glycolipids of microbial or endogenous origin in the context of the MHC-like molecule CD1d.\textsuperscript{359-361} The first ligand identified for NKT cells, \(\alpha\)-GalCer, was isolated from the marine sponge \textit{Agelas mauritianus} in a screen for bioactive substances,\textsuperscript{362,363} and was found to have anti-tumor effects in mice.\textsuperscript{364-366} This CD1d-binding glycolipid remains one of the most potent activators of NKT cells yet described.

As their name suggests, NKT cells exhibit a phenotype with features of both NK cells and T cells. Classification is based on expression of TCR and NK markers such as NK1.1 in the mouse, or CD161 and CD94 in humans, or binding of fluorescent CD1d tetramers loaded with \(\alpha\)-GalCer.\textsuperscript{367,368} Mature NKT cells in a resting state express markers associated with activated or memory T cells, including an intermediate expression of the TCR, high CD44 and CD69, and low expression of CD62L\textsuperscript{357,359} and CD40L.\textsuperscript{370,371} In mice, injection of \(\alpha\)-GalCer rapidly activates NKT cells, with reciprocal interactions in turn inducing maturation of DCs, typified by up-regulated expression of MHC and co-stimulatory molecules.\textsuperscript{354,355,372} The interaction also drives DCs to release significant quantities of IL-12 into the serum,\textsuperscript{373,374} primarily from the CD8\(\alpha^+\) subtype,\textsuperscript{223,375} which, as noted earlier, is also specialized for cross-presentation. Thus, when \(\alpha\)-GalCer is co-administered with a protein antigen, the NKT cell response induced provides excellent adjuvant activity, increasing the number of antigen-specific IFN-\(\gamma\)-producing CD4\(^+\) T cells and licensing potent antigen-specific CD8\(^+\) T cells with cytotoxic capacity.\textsuperscript{354,355} This T cell response requires CD40 signaling,\textsuperscript{354,372} with additional stimulation provided through up-regulated expression of CD70 on DCs engaging CD27 on T cells\textsuperscript{376} and Ox40/Ox40L interactions.\textsuperscript{377} It remains to be established whether such a licensing role is involved in host responses to tumors. In fact, NKT cell-deficient mice are more susceptible to carcinogen-induced tumors, implying a role for these cells in immunosurveillance.\textsuperscript{378} However, NKT cells also exhibit NK cell-like cytotoxic activities against tumors, with killing mechanisms including release of perforin/granzyme, Fas/FasL, and release of TRAIL.\textsuperscript{379-381} Also, when triggered with ligands such as \(\alpha\)-GalCer in preclinical models, NKT cells have been shown to limit tumor growth by the indirect effect of cytokine release, particularly the release of copious quantities of IFN-\(\gamma\), which has anti-angiogenic properties.\textsuperscript{382-384} Attempts to activate NKT cells with \(\alpha\)-GalCer in cancer patients have tended to focus on driving these direct and indirect tumor-killing activities, rather than on specifically exploiting the role of DC licensing. However, it is intriguing to note that an improved CD8\(^+\) T cell response to a chronic CMV infection was observed in one patient injected with \(\alpha\)-GalCer-loaded DCs, while another had a heightened CD8\(^+\) T cell response to an influenza vaccine given mid-treatment.\textsuperscript{385} Activation of NKT cells may therefore have provoked improved function in APCs presenting viral antigens. An immunization strategy in which \(\alpha\)-GalCer is co-administered with tumor antigens may very well be worth investigating. Our own preclinical data, and those of others, have shown vastly superior anti-tumor activities using DC-based vaccines loaded with \(\alpha\)-GalCer and specific antigens or peptides or whole irradiated tumor cells. Potent anti-tumor responses have also been elicited with protein or peptide antigens or whole irradiated tumor cells simply co-administered intravenously with \(\alpha\)-GalCer.\textsuperscript{354,355,386}

C. NK Cells

NK cells are effectors of innate resistance capable of lysing cells without prior sensitization.\textsuperscript{387} Like
NKT cells, NK cells engage in cross-talk with DCs, such that DCs can support the tumorcidal activity of NK cells, while cytokine-preactivated NK cells can induce DC maturation and cytokine production. These effects are cell-contact dependent and involve IFN-γ and TNF-α. The outcome of these interactions appears to be related to the ratio of NK cells to DCs. When ratios are low, DC maturation ensues, whereas when NK cell numbers are in significant abundance, DCs succumb to direct cytotoxic activity. Regardless, studies have shown that NK cells are indeed capable of licensing DCs to promote CTL responses against pathogens and tumor antigens. In vitro, the licensing of DCs by NK cells to induce lymphoma-specific CTL was shown to invoke IL-18 production by DCs, which may improve CTL function and provide positive feedback to NK cells. In fact, IL-18 has been shown to drive a distinct “helper” differentiation pathway of human CD56+ CD3- NK cells into CD56+CD83+CCR7+CD25+ NK cells, with features such as increased migratory responsiveness to lymph node-associated chemokines, a heightened capacity to produce IFN-γ, an ability to promote IL-12p70 production in DCs, and an ability to promote development of Th1 responses in vitro. In contrast, IL-2 selectively promotes cytotoxic function in NK cells that may limit their ability to promote the DC-mediated induction of Th1 responses.

Further underlining the complexity of DC-NK cell interactions, it has been shown that immature, as opposed to mature, DCs are more susceptible to NK-cell-mediated killing by virtue of their lower levels of MHC-I expression. It has been further proposed that NK-cell-mediated removal of mature DCs with less than optimal MHC expression prevents the generation of low-affinity T cells, a process termed “editing.” Thus, the fate of DCs that are used in vaccination protocols in cancer patients and, ultimately, the quality of T cell responses induced, may be determined by these complex interactions with NK cells. In this context, it is worth noting that DC injection in mice results in the accumulation of NK cells in the draining lymph nodes, which can be enhanced with some immune adjuvants. Where the adjuvant induced significant NK cell recruitment, the Th1 response induced was significantly increased over adjuvants in which no NK recruitment was observed. These studies underline the need to consider the interface between DCs and effector cells of the innate system, such as NK cells and NKT cells, in defining the outcome of immunotherapy protocols.

VIII. TRANSFER OF ANTIGEN BETWEEN DENDRITIC CELLS

Much of the literature on DCs describes a common paradigm, typically attributed to LCs, in which DCs located within the periphery respond to danger stimuli by migrating to the draining lymph nodes where, as mature cells, they present acquired antigen to T cells. In fact, applying this paradigm to DCs as a whole may be rather overstated. First, as highlighted earlier, many DCs actually function as resident cells in the lymphoid tissues, strategically located within these tissues to acquire antigens and associated danger signals directly from the lymph or blood. Second, it is becoming apparent that the cells that initially acquire antigens are not necessarily the same cells that engage T cells, implying transfer of antigens. The transfer of antigens between cells has been reported following administration of antigen-loaded bone marrow-derived-DCs, with antigens ending up in lymphoid resident DCs, Transfer of antigens has also been suggested for LCs and dermal dendritic cells draining the skin following murine herpes simplex virus infection, with T cell responses ultimately stimulated by lymph node-resident CD8α+ dendritic cells. A similar division of labor between migrating and resident DC subsets has been suggested to be operating in lymph-nodes draining the lung following influenza infection. It remains possible that viruses drive the antigen transfer by killing infected migratory DCs. However, if the phenomenon antigen-transfer from one DC subset to another proves to be applicable in other situations, then this would mean a significant re-assessment of the fundamental requirements for T cell activation. In terms of tumor antigen presentation, lymphoid resident DCs may not simply acquire antigen from tumor cells and debris draining to the lymph node, but as cargo transferred from migrating DC subsets. It is also possible that optimal T cell stimulation may require antigen presentation by more than one DC subset, as has been described
for CD4+ T cell responses to antigens from the skin.\textsuperscript{402,403} Most importantly, the phenotype of the recipient DC population becomes critical in driving an effective immune response. In the case of an infection such as murine herpes simplex virus, it is possible that infection-driven activation of recipient DCs ultimately drives the T cell response. In other situations, effective immunity may be reliant on circulation of factors capable of triggering activation of DCs within the lymphoid tissue. In the absence of these stimuli, regardless of the activation state of the migratory DCs, resident DCs may fail to stimulate immunity due to low co-stimulation or, perhaps worse, induce T-regulatory cells that actively suppress immunity. This may be particularly relevant in the tumor situation, where there is likely a dearth of suitable activation stimuli circulating to the lymphoid tissues and, indeed, exposure to suppressive factors is more commonly observed.

**IX. EXPLOITING THE DENDRITIC CELLS IN THERAPY**

It is now clear that a major hurdle to tumor immunity is the immunosuppressive environment generated by the tumor itself, and the direct and indirect effects that this suppression has on DCs. In attempt to negotiate around this barrier, many studies have focused on injecting cancer patients with ex vivo-generated DCs loaded with tumor antigens,\textsuperscript{404-406} thereby removing the DCs from the suppressive environment of the host. While these trials have demonstrated that injection of DCs can increase the numbers of T cells recognizing tumor antigens in the circulation and in the tumor, the overall clinical efficacy of this strategy has been low.\textsuperscript{407} However, a number of studies have reported durable, complete responses that were achieved with little or no associated toxicity.\textsuperscript{404,405,408,409} Furthermore, a commercial DC-based vaccine loaded with prostatic acid protein has shown promise in late-stage trials for the treatment of advanced prostate cancer.\textsuperscript{410} These promising results provide justification for investigating this therapy further. Although the actual number of circulating antigen-specific T cells required for an effective immunization strategy in cancer patients is unknown, it is generally recognized that the DC-based vaccination technology tested in cancer patients is far from optimal, especially compared with T cell responses induced naturally to infection. Improvements are therefore required to induce more powerful responses in a broader range of patients.

While acknowledging the low clinical responses rate of DC-based vaccines in the clinic, it is important to recognize that these clinical studies have directed focus on some important basic questions that will benefit the tumor immunotherapy field as whole; specifically, what is the best source of useful antigen(s), and what factors provide effective DC activation?

**A. Defined Antigens versus Whole-Tumor Preparations**

Tumor antigens used in DC-based therapies can be roughly grouped into two categories: well-defined tumor antigens and whole-tumor cell preparations in which the antigens are largely unknown. The advantages of using molecularly defined tumor antigens are that the DCs can be loaded with high concentrations of antigen, which may be necessary for efficient cross-presentation, and that the same antigen preparation can be used in different patients, making scale-up possible. Knowing the structure of the antigen also enables monitoring of specific vaccine-induced T cell responses using MHC tetramer technology or similar antigen-based assays. On the other hand, immunizing with only one, or a few, antigens increases the risk of the tumor developing antigen-loss variants, thereby avoiding T cell killing. In contrast, loading DCs with whole-tumor cells or tumor cell lysates theoretically enables T cell reactivity against several tumor antigens, minimizing the risk associated with antigen loss. In addition, some of these antigens will be patient-specific TSAs, effectively neo-antigens to which no T cell tolerance mechanisms will be operating.\textsuperscript{414} The real challenge of the whole-tumor approach is obtaining sufficient tumor material to manufacture vaccines that present physiologically relevant levels of antigen.

What information can be exploited to improve selection of antigens to load DCs before injection? In many studies only defined MHC class I-binding peptide epitopes were used. In fact, this strategy is unlikely to provide long-lived CTLs,
as it is now known that this requires CD4+ T cell help. It is therefore important that both MHC class I and class II-binding epitopes are loaded onto the same DC to ensure that licensing takes place in vivo. The use of whole-protein antigens to load DCs may circumvent this problem, with the added advantage that it is not necessary to know the structure of MHC molecules expressed by a given individual before treatment. However, the efficiency of cross-presentation in vitro is likely to be a limiting factor. A strategy to increase cross-presentation is the use of antibody-opsonized antigens (“immune complexes”), which will be internalized via FcRs, with the added benefit that appropriately targeted FcRs will activate the DCs.115,116,327,331 In fact, ex vivo loading of DCs may be the best way to exploit FcR-mediated uptake, because immune complexes may be otherwise efficiently targeted for destruction by macrophages and granulocytes in vivo.327 Using such a strategy may also increase the uptake of whole-tumor preparations, potentially reducing the problem of acquiring sufficient quantities of autologous tumor for vaccine manufacture. Studies already discussed in this review relating to providing “eat me” signals may yet provide strategies for dramatically increasing the uptake of whole-tumor preparations, with the added advantage that they may be associated with provision of endogenous danger ligands to activate the DCs. For example, human DCs loaded with heat-treated mesothelioma cells were shown to be more efficient at cross-priming naive human CTLs in vitro than DCs loaded with unheated mesothelioma cells, and this was attributed to enhanced levels of HSP70 in the treated tumor cells.411 Other simple procedures that encourage tumor cell injury, such as cryoablation and radiation treatment, may be useful in this regard.

A recent review examined the outcomes of a wide range of active immunotherapy trials (not just DC-based) using molecularly defined antigens versus strategies using whole autologous or allogeneic tumor cells. The responses of 3444 patients in 173 published trials was examined. Objective clinical responses were seen in 8.1% of patients treated with immunotherapies that utilized whole tumors or tumor extracts as antigens, compared with 3.6% when molecularly defined antigens were used. Thus, a statistically significant advantage to the whole-tumor strategy was observed.407 In practice, the two different approaches actually provide quite complementary information that can help in the development of active immunotherapy as a practical therapy. Analysis of clinical responders to whole-tumor vaccines can be used to determine ideal antigens to target by the defined-antigen approach. Because this approach permits more effective monitoring of induced responses, important information on maximizing vaccination formulation can be more readily addressed and fed back into the whole-tumor approach.

B. Ensuring Dendritic Cell Activation

A number of different regimens have been used to stimulate the injected DCs prior to injection. These include monocyte-conditioned medium to induce maturation, a cocktail of cytokines (IL-1β, TNF-α, IL-6, and prostaglandin E2) that mimic monocyte-conditioned medium, or the use of TLR ligands to stimulate activation.404,406,412 The inclusion of TLR ligands may have the added advantage of encouraging NK recruitment to the draining lymph node, thereby potentially invoking the licensing role of these innate cells. Considering the role of CD40-CD40L interaction in licensing the DCs, CD40L has been used in combination with the above regimens.405,413 As noted above, the inclusion of MHC class II-binding peptides is required to recruit CD4+ T cell help, but a more useful strategy may be to load the DCs with the NKT cell ligand α-GalCer, which may function as a universal helper epitope due to the fact that its restriction element, CD1d, is non-polymorphic.

C. Dendritic Cell-Based Vaccines that Stimulate Resident Cells

In our opinion, the possibility that antigen is transferred between DC populations has important implications for the design of vaccine-based therapy. In DC-based therapy in particular, it may not be sufficient to concentrate solely on activating the injected DCs, as has been the case in therapies to date; some facility for improving the activation status of the resident cells must also be considered. Our own studies highlight the importance of such a strategy. When a vaccine consisting of OVA-loaded BM-DCs was injected intravenously into
mice, an OVA-specific CD8+ T cell response was initiated. Stimulating the injected DC with TLR ligands before injection had only a limited impact on the induced response. However, simultaneous injection of the DC vaccine with a TLR ligand resulted in a significant enhancement of the CD8+ T cell response. Further analysis showed that a resident population of langerin+ CD8α+ DC was responsible for the enhanced response, because specific depletion of this cell type reduced the CD8+ T cell response to levels achieved with DC vaccine alone. The injected TLR was therefore improving the stimulatory capacity of these resident cells rather than the injected cells, which was confirmed when enhanced T cell responses were also observed when TLR-deficient DCs were injected (Petersen et al., unpublished results).

Another strategy to enhance function of resident DCs is the incorporation of α-GalCer into DC vaccines. We have found that both α-GalCer and protein antigens can be transferred to resident DCs in vivo. Thus, the injected and recipient cells are licensed by interaction with NKT cells, allowing them both to participate in the induced antigen-specific CTL response (Petersen et al., unpublished results). Evidence of transfer of α-GalCer between cells has been also reported following injection with α-GalCer-loaded tumor cells, with resident DCs again participating in the induction of specific CTL.\(^{414,415}\) The activation of NKT cells with α-GalCer in vivo has also been shown to provoke differentiation of MDSCs into APCs with high levels of CD86 and CD40. Not only does this process provide relief from the suppressive function of MDSCs, but the differentiated cells are able to prime tumor-specific CD8+ T cells.\(^{414,415}\) This “activation” of MDSC is dependent on the presentation of α-GalCer by the MDSC and CD40-CD40L interactions.\(^{414}\) Whether the injection of α-GalCer-loaded DCs results in the transfer of the glycolipid to MDSCs to drive differentiation remains to be demonstrated. While these preclinical data suggest a significant adjuvant effect of incorporating NKT cell ligands such as α-GalCer into vaccines to drive T cell-mediated immunity, to date, no published clinical studies have deliberately set out to exploit this function.

D. In Vivo Targeting of Dendritic Cells

The process of culturing DCs ex vivo for the purpose of generating autologous vaccines can be laborious and expensive, and requires specialized laboratories to undertake processing under good manufacturing practice guidelines. For this reason, considerable research activity has been directed at strategies to target antigens directly to DCs in vivo, with the aim of developing reagents that can be produced in bulk quantities. In addition, the opportunity to target specific DC subsets that are specialized in presentation may result in particularly efficient induction of T cell responses. These strategies typically use antibodies to target uniquely expressed cell surface receptors. For example, initial attempts to target APCs as a broad group was based on their expression of MHC class II molecules. Antigens conjugated to anti-MHC class II antibodies,\(^ {416-419}\) or recombined with anti-MHC class II antibodies (referred to as “Troybodies”)\(^ {420}\) were used to elicit T cell responses in vivo. Similarly, the capacity of superantigens to bind MHC class II molecules has been exploited to target antigens to APCs,\(^ {225}\) with the added advantage that this method appears to promote cross-presentation. However, many MHC class II-positive cells are not desirable APCs, so more specific targeting of DCs is required. In the mouse, this has been achieved by conjugating antigen to antibodies against CD11c\(^ {418}\), even though CD11c is often regarded as a DC marker, it is expressed on other cell types including NK cells and activated T cells. Perhaps some of the most useful surface receptors to target are members of the CLR family, because, as noted earlier, a number of these are specifically expressed by DC subsets. In humans, progress has been made in developing reagents to target DC-SIGN. A humanized antibody has been engineered, and can target conjugated antigens to monocyte-derived DCs in vitro, eliciting specific responses from naïve and memory T cells.\(^ {322}\) In another study, administration of anti-DC-SIGN antibodies carrying either tetanus toxoid peptides or keyhole limpet hemocyanin to Rag2\(^ {–}\)/Cig mice reconstituted with human immune cells induced antigen-specific human T cells without additional adjuvant requirements.\(^ {421}\) One of the more appealing CLR targets is Clec9A, because this is expressed by BDCA-3+ human DCs, the putative
human equivalent of murine CD8α+ DCs specialized in cross-presentation. Using some of the above strategies to target antigens to steady-state, non-activated DCs has actually provided some of the most compelling evidence that tolerance mechanisms are invoked by DCs unless an activation signal is provided. Given that tumors also deliberately create a suppressive environment, it is clear that therapies based on in vivo targeting of DCs require some form of activation signal, such as a TLR ligand or anti-CD40. The use of α-GalCer should also be considered, as it has proven to be a useful adjuvant in mouse studies.

X. CONCLUDING REMARKS

As we learn more about DC function, it appears that their stimulatory capacity is determined by multiple interactions that may work in additive, or even synergistic, fashion. These signals define the initial response to danger, determine the process of acquisition of antigens, and influence the different presentation pathways antigens access. There are also influences on the stimulatory molecules expressed on the cell surface to drive adaptive responses. Thus, DCs can be triggered in many different ways, with potentially significant implications for the responses they induce. This is made even more complex by the existence of different DC subsets with specialized functions. While the evidence that DCs can acquire and present tumor antigens is mounting, the fact is, responses induced naturally in cancer patients are usually a case of “too little, too late.” Using DC-based vaccines therapeutically to enhance these responses has been a fruitful exercise in gaining knowledge about immune processes in vivo, but is probably as yet too unsophisticated to extract the full potency of the immune system. By taking into account the myriad of factors we now know are involved in driving DC function, we may yet be able to induce more effective responses. There is therefore still worthwhile territory to cover in this approach. This same knowledge may be used to generate a second wave of useful vaccines based upon in vivo targeting of DCs. It is certainly not unreasonable to expect DCs in their natural state to have a greater stimulatory potential than their in vitro-cultured counterparts, particularly when immunosuppressive factors released by the tumor have been neutralized. Research into adjuvants capable of providing effective in vivo activation stimuli should therefore be actively pursued, be they compounds that directly stimulate DCs or that work indirectly through stimulating other innate cells. Optimization of these protocols may provide the best way to harness the natural stimulatory properties of DCs.

ACKNOWLEDGEMENTS

The authors are supported by the New Zealand Health Research Council and the Cancer Society of New Zealand. I.F.H. was supported by a New Zealand Health Research Council Sir Charles Hercus Fellowship.

REFERENCES

Tumor Antigen Presentation by Dendritic Cells


Tumor Antigen Presentation by Dendritic Cells


100.

Critical Reviews™ in Immunology


Tumor Antigen Presentation by Dendritic Cells


249. Song R, Harding CV. Roles of proteasomes, transporter for antigen presentation (TAP), and beta 2-microglobulin in the processing of bacterial or particulate antigens via an alternate class I MHC processing pathway. J Immunol. 1996;156:4182–90.


Tumor Antigen Presentation by Dendritic Cells


Tumor Antigen Presentation by Dendritic Cells


Tumor Antigen Presentation by Dendritic Cells


The Role of Chemokines in Migration of Metastatic-like Lymphangioleiomyomatosis Cells

Gustavo Pacheco-Rodriguez and Joel Moss*

Translational Medicine Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD

*Address all correspondence to Joel Moss, MD, PhD, NHLBI, National Institutes of Health, Building 10, Room 6D05, MSC 1590, Bethesda, MD 20892-1590; Tele.: 301-496-1597; Fax: 301-496-2363; Email: mossj@nhlbi.nih.gov.

ABSTRACT: Lymphangioleiomyomatosis (LAM), a rare cystic lung disease with multi-organ involvement, occurs primarily in women of childbearing age. LAM can present sporadically or in association with tuberous sclerosis complex (TSC). Loss of lung function in patients with LAM can be attributed to the dysregulated growth of LAM cells, with dysfunctional TSC1 or TSC2 genes, which encode hamartin and tuberin, respectively, leading to hyperactivation of the mammalian target of rapamycin (mTOR). LAM cells are smooth muscle-like cells that express melanoma antigens such as gp100, a splice variant of the Pmel17 gene. Tuberin and hamartin form heterodimers that act as negative regulators of mTOR. Lack of TSC2 function, as occurs in LAM cells, leads to the production of the chemokine CCL2/monocyte chemotactic protein 1 (MCP-1), which increases LAM cell mobility. Although many chemokines and their receptors could influence LAM cell mobilization, we propose that a positive-feedback loop is generated when dysfunctional TSC2 is present in LAM cells. We identified a group of chemokine receptors that is expressed in LAM cells and differs from those on smooth muscle and melanoma cells (Malme-3M). Chemokines have been implicated in tumor metastasis, and our data suggest a role for chemokines in LAM cell mobilization and thereby in the pathogenesis of LAM.

KEY WORDS: cell motility, chemokines, chemokine receptors, cystic lung disease, lymphangioleiomyomatosis, metastasis, mammalian target of rapamycin, smooth muscle cells, tuberous sclerosis complex

I. INTRODUCTION

Lymphangioleiomyomatosis (LAM) is a rare cystic lung disease that is primarily found in women of childbearing age. LAM presents with progressive dyspnea, wheezing, cough, recurrent pneumothoraces, chylothorax, abdominal hemorrhage, involvement of the axial lymphatics (e.g., lymphangioleiomyomas), and abdominal tumors (e.g., renal angiomyolipomas).1,2 LAM occurs sporadically or in association with tuberous sclerosis complex (TSC), an autosomal dominant syndrome of variable penetrance, which is characterized by hamartoma-like tumor growths (e.g., facial angiofibroma, ungual fibroma, shagreen patch, renal angiomyolipoma, and pulmonary LAM nodules) and neurological disorders.3 These hamartomatous tumors are believed to be of mesenchymal origin

ABBREVIATIONS

Deptor, DEP-domain-containing mTOR interacting protein; LAM, lymphangioleiomyomatosis; mTOR, mammalian target of rapamycin; mLST8/GβL, mammalian lethal with Sec 13 protein 8; mSIN1, mammalian stress-activated protein kinase interacting protein 1; MCP-1, monocyte chemotactic protein 1; MMP, matrix metalloprotease; PRAS40, proline-rich AKT substrate 40 kDa; Protor-1, protein observed with Rictor-1; Raptor, regulatory-associated protein of mTOR; Rheb, Ras homolog enriched in brain; Rictor, rapamycin-insensitive companion of mTOR; TSC, tuberous sclerosis complex
and are classified as perivascular epithelioid cell neoplasms.\(^4\)

LAM cells are phenotypically smooth muscle-like cells that express melanoma antigens such as gp100, CD63/LAMP-3, Melan-A, and MART1, and have abnormalities in the \(TSC1\) or \(TSC2\) genes.\(^5\) Loss of TSC gene function results in hyperactivation of the mammalian target of rapamycin (mTOR).\(^6\) LAM is believed to involve the migration of LAM cells between organs, and therefore we designated these events as a metastatic-like process. There is evidence that LAM cells disseminate by lymphatic and hematogenous but not transcoelomic routes. After single-lung transplantation, LAM cells from the recipient were shown to colonize the transplanted lung, suggesting a metastatic process.\(^7,8\) Consistent with lymphatic and hematogenous spread, LAM cells can be detected in blood, urine, expectorated chyle, and pleural and abdominal chylous fluids.\(^9\)

Metastatic cells are capable of translocation to target sites. Cell motility can be directed by gradients of chemokines that interact with specific receptors on the plasma membrane of tumor cells.\(^10\) Metastatic cells migrate to specific sites distant from the primary tumor growth and “home” to an appropriate environment described as “soil,” which appears to be identified by specific soluble chemoattractants produced by cells at the metastatic site.\(^11,12\)

Chemokines could be produced in response to multiple factors. Infections are one of the best-characterized processes in the recruitment and homing of immune cells, as are inflammation and tissue injury. Chronic inflammation is a characteristic of many cancerous processes that lead to the activation of pathways involving nuclear factor \(\kappa B\) participation in the transcription of chemokines that attract cancer cells to sites of metastasis.\(^10,12\)

To understand the molecular events that lead to LAM cell growth and dissemination, we investigated the potential role of chemokines and their receptors in the spread of LAM cells.

## II. LAM CELLS AND LAM LUNG NODULE

The cells responsible in LAM are termed “LAM cells,” spindle- and epithelioid–shaped smooth muscle-like cells that contain dysfunctional \(TSC2\) or \(TSC1\) genes and form part of lung nodular structures, which express melanoma as well as smooth muscle cell antigens.\(^2,5\) Whereas LAM cells of both phenotypes synthesize smooth muscle-cell proteins (e.g., smooth muscle \(\alpha\)-actin, vimentin, desmin), the epithelioid cells appear to produce gp100, a premelanosomal protein product of alternatively spliced \(Pmel17\) transcripts. MART-1, CD63, and PNL2 are all melanosomal proteins controlled by microphthalmia transcription factor and produced in a group of pathological mesenchymal-derived cells characterized as perivascular epithelioid cells.\(^13\) Nodular LAM structures are covered with hyperplastic type II pneumocytes and contain mast cells and mast cell products (e.g., chymase). Cells lining lymphatic channels within the nodules react with antibodies against lymphatic endothelial cells.\(^14\)

The roles of \(TSC1/2\) have been defined previously: \(TSC2\) gene on chromosome 16p13.\(^3\) and \(TSC1\) gene on chromosome 9q34 encode tuberin and hamartin, respectively. Tuberin and hamartin form heterodimeric complexes that negatively regulate the mTOR serine-threonine kinase. Multimeric complexes containing mTOR termed mTORC1 and mTORC2 are central to cell growth, proliferation, gene transcription, and protein synthesis.\(^15\) mTORC1 is a multiprotein complex comprised of five proteins sensitive to rapamycin: mTOR, Raptor (regulatory-associated protein of mTOR), mLST8/GrbL (mammalian lethal with Sec 13 protein 8), PRAS40 (proline-rich AKT substrate 40 kDa), and Deptor (DEP-domain-containing mTOR-interacting protein). The mTORC2 complex is less sensitive to rapamycin and contains six proteins: mTOR, Rictor (rapamycin-insensitive companion of mTOR), mSIN1 (mammalian stress-activated protein kinase interacting protein 1), Protor-1 (protein observed with Rictor-1), mLST8, and Deptor.\(^16\) Components of TORC1 (i.e., Raptor), and TORC2 (i.e., Rictor) regulate the different functions attributed to mTOR.\(^15\) mTORC1 activity is largely regulated by the GTPase Rheb (Ras homolog enriched in brain), which is a substrate for the GTPase-activating function of tuberin (Fig. 1).\(^17-19\)

Akt-dependent signaling regulates mTOR, and thereby phosphorylation of S6K1, S6K2, and eIF4E. Phosphorylation of tuberin by Akt causes its inactivation and disassembly of the TSC1/TSC2 complex;\(^18\) AMP-dependent protein kinase regulated
by the tumor suppressor LKB1 also modulates the function of the TSC1/TSC2 complex (Fig. 1).

III. MOLECULAR ASPECTS OF LAM CELL METASTASIS

Identification of phenotypically and genotypically similar cells in kidneys, lymphatics, and lungs of LAM patients suggest that LAM cells disseminate in a metastatic-like process. Because LAM cells are able to invade transplanted lungs, it was suggested that hematogenous and lymphatic routes are used for their dissemination. Cells that metastasize by lymphatic and hematogenous routes appear to begin with detachment from the primary locus and invade the local tissue stroma, followed by penetration into local blood or lymphatic vessels and transit to arrest points after surviving the circulatory system. Arrested cells penetrate the parenchyma at a metastatic site, adapt to the environment, and proliferate. All of these events are required for metastatic cell proliferation at a distant site, but the intercellular communication among these cells and receptive tissues plus additional cues (e.g., chemokines) to mobilize and anchor cells at metastatic sites are also important.

IV. CHEMOKINES AND CHEMOKINE RECEPTORS

Chemokines were initially identified as molecules that induce the migration of leukocytes and are produced by immune cells that respond to inflammatory responses. The lung responds to environmental insults by recruiting inflammatory cells that secrete chemokines that attract other cells to the lung; thus, chemokines may have roles in both innate and adaptive immunity. Chemokines also participate in organ homeostasis and in cancerous processes. Many cells of non-immune origin not only have chemokine receptors, but also synthesize and secrete chemokines.

Four subfamilies of chemokines (C, CC, CXC, and CX3C) are defined by the location of cysteine in their primary structure. There are at least 53 chemokines, secreted proteins of 66 to 111 amino acids, except for CXCL16 and CX3CL1, which are membrane-bound proteins. Genes that encode chemokines are present on different human chromosomes, with clusters of chemokine genes on chromosomes 4q21.1, 17q11.2, and 17q12. Chemokines are also classified into functional subfamilies, including: inflammatory chemokines, which participate in innate immunity (e.g. CXCL16, CXCL1), extravasation (CX3CR1), and adaptive immunity (e.g., CCL2, CCL6, CCL27, CCL28); and homeostatic chemokines, which participate in hematopoiesis (CXCL12), follicular activities (CXCL13), T lymphopoiesis (CXCL19 and CCL21), and T-cell–dendritic cell interaction (CCL18).

Chemokine receptors are seven-transmembrane-domain, G protein-coupled receptors of the G family and members of the rhodopsin-like seven-transmembrane superfamity. Binding of chemokines to their receptors causes conformational changes and, in some cases, receptor dimerization, which affects guanine nucleotide exchange on the alpha subunit of the G–protein complex (Gαβγ), leading to the formation of Gα-GTP and its dissociation from G-βγ. The individual units activate specific signaling pathways in which small GTPases such as Rho, Cdc42, and Rac affect cell motility. Gβγ subunits activate phospholipase C, resulting in the production of inositol triphosphate and diacylglycerol. Thus, the multiple effects of chemokines could lead to LAM cell invasiveness and migration due to the activation of the GTPases Rac1 and Rho. Notably, the Rho protein is also regulated by the TSC2 gene product tuberin.
V. LAM AND CHEMOKINES

Concentrations of CCL2 (MCP-1), CXCL1 (GRO1), and CXCL5 (ENA-78) in bronchoalveolar lavage fluid from patients with LAM were higher than in that from healthy volunteers, suggesting that these chemokines could be involved in the recruitment of LAM cells to the lung (Fig. 2). Levels of CCL2/MCP-1 were higher in bronchoalveolar lavage fluid from LAM patients than in that from healthy volunteers.

Polymorphisms of the CCL2/MCP-1 gene were more frequent in patients with LAM than in healthy volunteers, and were correlated with rates of decline in lung function. Frequencies of two polymorphisms in the promoter of the CCL2/MCP-1 gene were compared in LAM patients and age- and sex-matched healthy volunteers, and differed significantly in the two groups. The frequency of AA at positions −2578 and −2136 was greater in LAM patients than in volunteers, and more detailed analysis suggested that the CCL2/MCP-1 gene and protein may be genetic modifiers in the development of LAM.

CCL2/MCP-1 selectively attracted cells with dysfunctional TSC2. CCL2/MCP-1 was further shown to be associated with LAM nodules in about 70% of patients. As part of a potential feed-forward pathway, TSC2 regulated CCL2/MCP-1 production in human skin and mouse cells. CCL2 was overexpressed in human cells grown from TSC skin, perungual fibroma, and angiofibroma. Overexpression of MCP-1 in mouse Tsc2−/− cells was mTOR dependent and resulted from a loss of tuberin function. High levels of CCL2/MCP-1 were reduced in both human and rodent cells lacking tuberin function, but reconstitution of cells with tuberin or treatment with rapamycin to abrogate mTOR activity decreased the production of CCL2/MCP-1. These data also suggest that TSC2 could down-regulate CCL2/MCP-1 production directly or indirectly by the action of mTOR. Altogether, it is proposed that CCL2/MCP-1 is involved in LAM cell dissemination by a paracrine feedback loop (Fig. 1).

VI. LAM AND CHEMOKINE RECEPTORS

LAM cells within lung lesions displayed a unique group of chemokine receptors, including CCR2, CXCR4, CCR7, and CCR10, which allowed grouping of the LAM samples from smooth muscle or melanoma cells. Global gene-expression...
sion analysis of microdissected LAM lung cells of patients with sporadic LAM identified a set of transcripts that distinguished lung LAM cells from smooth muscle and melanoma cells (GEO database GSE12027). Some chemokines were differentially expressed and may participate in attracting and anchoring cells to sites of metastasis.

Among the receptors most frequently found in diverse cancers are CXCR4, CCR7, CCR4, CCR10, and CXCR7. Metastatic breast cancer cells express the chemokine receptors CXCR4 and CCR7, whereas malignant melanoma cells express CCR10, CXCR4, and CCR7. Smooth muscle-like LAM cells in more than 50% of patients reacted with antibodies against the chemokine receptors CCR2, CCR7, CCR10, CXCR2, CXCR4, and CXCR1, which supports the conclusion that cancer cells exhibit a characteristic profile of chemokines and their receptors. Thus, cells can to some extent be identified by their chemokine/chemokine receptor molecular signatures, which seem to direct metastasis to specific organs. Although it is not clear why a single tumor cell has multiple chemokine receptors, the observation that chemokine receptors are able to homo- and heterodimerize to produce specific effects suggests this as a mode of chemokine/chemokine receptor action in LAM cells.

We determined which chemokine receptors were more common in LAM lung nodules (Fig. 2), and found that the receptor and ligand found most often in LAM nodules and known to be involved in cancer are CXCR4 and its ligand SDF-1 (CCL12). This receptor has been identified in at least 23 types of cancer, including breast and ovarian cancers and melanoma. CXCR4 and CXCR3 are functional receptors in melanoma cells and are involved in the activation of the MAPKs p44/42 and p38 pathways. We did not detect high levels of CCL12 in the bronchoalveolar lavage fluid of LAM patients, but a large percentage of LAM cells expressed CXCR4, and CXCR4 mRNA levels were high in LAM cells from lung nodules. We were unable to define the role of the CCL12-CXCR4 pathway in LAM cells, but angiomyolipoma cells appear to respond to CXCL12 by activating AKT and p42/44.

Some cells in LAM nodules express receptors such as CXCR6, CXCR5, CCR8, CCR5, CCR4, CCR3, and CCR1. CXCR6 and its ligand CCL16 are present in several cancers including liver, prostate, colon, breast, ovary, glioblastoma, lung, lymphoma, melanoma, and renal cell carcinoma, but not in thyroid or head and neck cancers. CXCL16 may be relevant to LAM because it stimulates secretion of IL-8 and IL-6, activation of Akt, p70S6, and initiation factor 4E, thereby affecting cell proliferation and invasion. Thus, activation of some pathways in LAM lesions could result from signaling cross-talk.

CCR2 and CCR10 are among the receptors found most frequently in LAM lungs. CCR10 has been identified as a receptor involved in melanoma metastasis. CCR2 is clearly involved in breast cancer, glioma, lung cancer, melanoma, and prostate cancer. We were unable to show that CCR2 is a receptor that distinguishes LAM cells from melanoma or smooth muscle cells, but both CCR2 and CCR10 bind CCL2/MCP-1, which is one of the chemokines most elevated in LAM.

**VII. MECHANISMS OF DYSREGULATION OF CHEMOKINES AND THEIR RECEPTORS**

Multiple factors could contribute to CCL2/MCP-1 levels in individuals with LAM, and the effects apparently differ from those in other interstitial lung diseases. Mechanisms that lead to dysregulated expression of chemokine and chemokine receptors in LAM patients are not established. It is known that infections, inflammatory responses, and hypoxia via co-receptors (e.g., HER2) can enhance the levels of chemokine receptors. Indeed, LAM lungs exhibit bronchiolitis, which could be a response to inflammation. In addition, the presence of hyperplastic type II pneumocytes surrounding the LAM cells might suggest an association with underlying inflammation or LAM cell products that could affect their proliferation.

The levels of CCL2 are regulated by multiple factors under conditions including menstruation and early pregnancy. These findings could be important to female LAM patients because of the putative role of estrogens in LAM pathogenesis. In support of this notion, 17-β-estradiol increased metastasis in both male and female mice engrafted with Tsc2−/− cells derived from Eker rats. Although all of these findings appear
congruent with the pathogenesis of pulmonary LAM, an effect of estrogens on LAM cannot be deduced because of the use of non-human cell lines. The fact that estrogens regulate synthesis of chemokines could be a mechanism for enhanced metastasis in women with sporadic LAM.

The effects of LAM cells on adjacent stromal cells are not clearly established. We found, however, that CXCL5/Gro-1, which has been implicated in tumor progression by causing senescence of tumor-associated fibroblasts,\textsuperscript{49,50} was elevated in bronchoalveolar lavage fluid from LAM patients.

**VIII. OTHER ASPECTS OF LAM CELLS**

Metastatic growth involves adhesion molecules (e.g., cadherins, integrins, immunoglobulins, proteoglycans, CD44), and proteolytic enzymes such as metalloproteases (MMPs) (e.g., MMP-1, MMP-2, MMP-3, MMP-7, MMP-9). The proteoglycan CD44 receptor for hyaluronan is present on LAM cells.\textsuperscript{51} CD44, encoded in 20 exons on chromosome 11, is present in many different types of cells. The splice variant CD44v6, which has been involved in the metastasis of various cancers, is present on LAM cells.\textsuperscript{51} Thus, proteins implicated in metastatic pathways are associated with LAM cells, conferring metastatic potential. CD44 binds osteopontin and is cleaved by metalloproteinases.\textsuperscript{52} LAM nodules contain MMP-2, MMP-9, MMP1, and MMP activators (MT1-MMP) and inhibitors (TIMPs).\textsuperscript{53,54} The presence of CD44v6 and MMPs support molecular aspects of LAM metastasis. In addition to providing a metastatic phenotype to LAM cells, CD44 could be involved in the metabolism of chemokines by interacting with chemokine scavenger receptors.

**IX. SUMMARY**

Chemokines such as those found at high levels in bronchoalveolar lavage fluid from LAM patients could be important in metastasis and modify the LAM microenvironment to affect cell–stroma interactions. LAM cell mobilization could be favored by the presence of specific chemokines and their receptors, making both ligand and receptor potential therapeutic targets. The selective attraction of LAM cells by CCL2/MCP-1 and overexpression of CCL2/MCP-1 by cells lacking the tumor suppressor tuberin lead us to propose a paracrine feedback loop of LAM cell motility in which this chemokine has a major role (Fig. 1).

**REFERENCES**

The Role of Chemokines in Migration of Metastatic-like Lymphangioleiomyomatosis Cells