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Rational Approaches to Immune Regulation

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Abstract

Our studies are mainly focused on developing strategies of immune regulation. In the case of infectious and neoplastic disease, our approach is to upregulate cell-mediated immunity to viral of tumor antigens using an intracellular bacterium as a vector for targeting these antigens to the major histocompatibility complex (MHC) class I and class II pathways of antigen processing, in addition to exploiting the adjuvant properties of the vector to stimulate innate immunity. In the area of autoimmunity, we are attempting to downregulate the immune response by specific immune intervention directed against autoreactive T cells. In these studies we use murine models for multiple sclerosis. Our approach is to use both rationally designed T cell receptor (TCR) peptide analogs and recombinant viral vectors that express TCR components to regulate the disease.

Key Words

Antigen processing
Cell-mediated immunity
EAE
Listeria monocytogenes
Tumor immunotherapy
Vaccinia

Introduction

Our laboratory is interested in the properties of proteins (both foreign and self) that render them immunogenic and how such immunogencity may be modulated in vivo. In the case of the immunoglobulin receptor on B cells, both the antigenic site on the protein antigen and the binding site of the immunoglobulin are topographic surfaces. However, in the case of the T cell receptor (TCR), the antigenic region of the protein is a peptide derived by cellular processing and expressed on the surface of an antigen-presenting cell associated with a molecule of the major histocompatibility complex (MHC). The cellular compartment in which the T cell epitope is generated determines whether it emerges at the cell surface bound to MHC class I or II molecules and, therefore, what type of T cell response is elicited. There have been enormous advances made in the last few years in our understanding of the molecular and cellular machinery that governs the presentation of antigens to the immune system. In our laboratory, we are attempting to apply this knowledge to the development of strategies of

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immune regulation for a number of disease models. Our work serves two purposes. The first is that we believe that we can learn much about the mechanisms of immunity by testing the outcome of applying current theories to manipulating immunopathological states. The second is to heed Albert Sabin's maxim "To yield to every whim of curiosity, and to allow our passion for inquiry to be restrained by nothing but the limits of our ability shows an eagerness of mind not unbecoming to scholarship. But it is *wisdom* that has the merit of selecting from among the innumerable problems which present themselves, those whose solution is important to mankind" *(1)*.

In the case of foreign antigens, we are attempting to enhance the immune response in the design of more effective vaccines against viral diseases and against tumor cells. To do this, we are using a facultative intracellular bacterium, *Listeria monocytogenes*, which has the unusual ability to live and grow in the cytoplasm of the cell. We have shown that recombinant forms of this organism, which have been transformed to express foreign antigens, are excellent vectors for targeting foreign antigens to the MHC class I pathway of antigen processing with the generation of authentic cytotoxic T lymphocytes (CTL) epitopes. In the area of autoimmunity, we are attempting to down regulate T cell responses by specific immune intervention directed against autoreactive T cells. In these studies, we use two murine models for multiple sclerosis. The first is the experimentally induced autoimmune disease known as experimental autoimmune encephalomyelitis (EAE). This disease of mice and rats can be caused by $CD4^+$ T cells reactive with myelin basic protein that use a restricted number of variable (V) genes to construct their receptors. Our approach is to use both rationally designed TCR peptide analogs and recombinant viral vectors that express TCR components to regulate the disease. Another murine model for multiple sclerosis is induced by a neurotropic strain of murine hepatitis virus. In this case, the cause of the postinfectious demyelinating event is unknown. Although cells of the immune system have been implicated indirectly, the identification of the phenotype of these cells has been elusive. In collaboration with Weiss' laboratory, we are attempting to determine the immune mechanism of demyelination. Although we hope our studies will allow us to design effective therapies for each disease state, in the process, we believe they may shed light on fundamental questions regarding the regulation of the immune response to protein antigens.

Upregulation of Immune Responses Using an Intracellular Vector for Poor Immunogens

In this project, we have been using an unusual intracellular bacterium to target antigens, that appear to be poorly immunogenic, to the immune system. The two major areas on which we have focused are HIV infections, where the normal immune response fails to induce sterilizing immunity, and cancer, where the antigens are often strongly homologous to self-antigens and suppressive mechanisms are also in effect.

Six years ago *(2)* we proposed that the type of immunity induced by the facultative intracellular parasite *L. monocytogenes* could be ideal for boosting the immune response to foreign antigens. The unusual property of *L. monocytogenes* to live in the cytoplasm of host cells (*see* Fig. 1) sequesters the bacterium from the humoral immune response and requires a strong cell-mediated immune response to resolve infection *(3)*. It has been shown that *L. monocytogenes* enters the host cell and is taken up in a phagosome but, unlike most other intracellular bacteria *(4)*, *L. monocytogenes* escapes from the phagosome into the cytoplasm of the cell by disrupting the phagosomal mem-

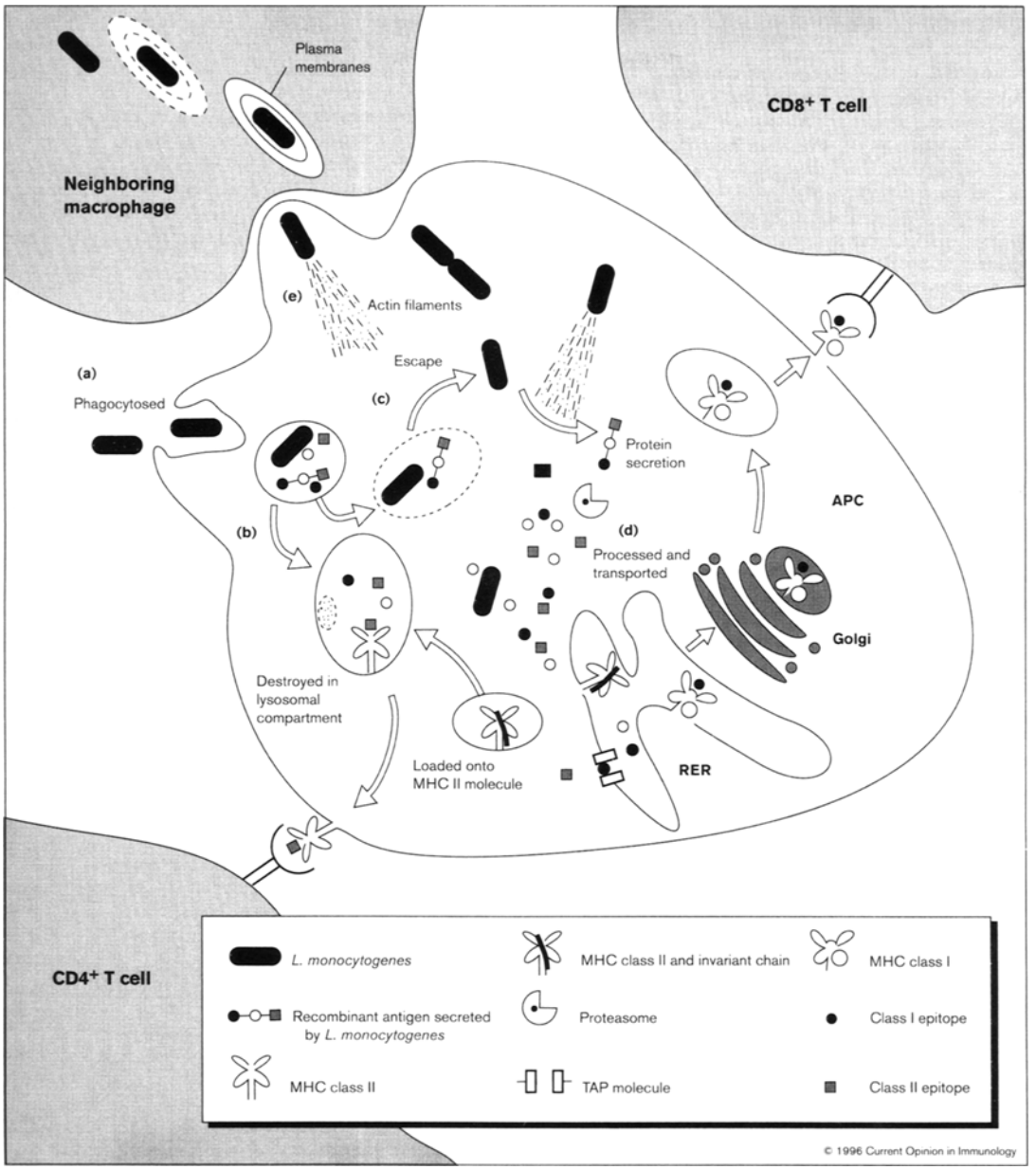

Fig. 1. How antigens secreted by *L. monocytogenes* access both the MHC class I and class II pathways for antigen processing. **(A)** After phagocytosis into an antigen presenting cell, *L. monocytogenes* is either **(B)** destroyed within a lysosomal compartment, where peptides may be loaded onto MHC class II molecules, or **(C)** it escapes into the cytoplasm of the cell. In the cytosolic compartment, *Listeria* can grow and any protein it secretes can be processed by proteosomes into peptides that will be transported **(D)** to the endoplasmic reticulum for loading onto MHC class I molecules. **(E)** In the cytosolic compartment, *Listeria* reorganizes the host cells actin into "tails," which propel it around the cell and to the cell's periphery. There it can be internalized by a neighboring cell, and by breaking through, two plasma membranes colonize this cell. Reprinted with permission from Current Biology Ltd., London, UK from Curr. Opin. Immunol. 8:664–669.

brane, primarily through the action of listeriolysin O. The bacteria replicate in the cytoplasm, and then move to the periphery of the cell where they form pseudopod-like structures, which are recognized and internalized by adjacent cells where the cycle is repeated *(5)*. The unusual ability of *L. monocytogenes* to escape phagolysosomal restriction and live in the cytoplasm explains why this bacterium is particularly effective as a vector for targeting the class I-restricted pathway of antigen processing. The localization of bacteria in the lysosomal compartment after invasion ensure that antigens expressed by this bacterium enter the MHC class II pathway for antigen processing, since the majority of bacteria are killed and digested in this compartment in vivo *(6)*. Finally, the ability of the bacterium to spread from cell to cell without entering the extracellular matrix explains its inadequacy in inducing an antibody response.

Early studies showed that $CD8^+$ T cells are the major effector subset in controlling and clearing the listerial murine infection *(7,8)*. Hemolytic activity of the bacteria is required for T cell induction in vivo and presentation to $CD8^+$ T cells in vitro *(9,10)*. The role of $CD4^+$ T cells appears to be less critical for the control of *L. monocytogenes* infections in the murine model. Depletion of $CD4^+$ lymphocytes during adoptive transfer has a much less pronounced effect than depletion of $CD8^+$ lymphocytes *(7)*. Depletion of $CD4^+$ cells during primary *L. monocytogenes* infection or in *L. monocytogenes* immune mice had only a marginal effect in controlling *L. monocytogenes* challenge, although delayed-type hypersensitivity and granuloma formation were severely diminished in mice treated with anti-CD4 antibody *(8)*. The strong DTH response to *L. monocytogenes* can now be directly related to the innate immune response to this bacterium that takes place early in infection *(11)*. Phagocytosis of *L. monocytogenes* by a resident macrophage results in the secretion of interleukin (IL)1, IL12, and tumor necrosis factor (TNF)α and its activation to a state that is competent to destroy the invading parasite by nitric oxide ($NO^{\cdot}$) production. IL1 secreted by resident macrophages activates neutrophils and helps to maintain the activated state of macrophages, which have increased MHC expression. IL12 and TNF act on NK cells stimulating them to secrete interferon (IFN)γ. This facilitates the expansion of Th0 cells, which under the influence of IL12 and IFNγ differentiate to the Th1 phenotype that secretes IL2, TNFα, and IFNγ. Later in infection, IL10 may be secreted by macrophages that can downregulate the activation of Th0 to Th1. These stages in innate immunity are consistent with the ability of *L. monocytogenes* to promote cell-mediated over humoral immunity and verify that the in vivo $CD4^+$ T cell response is also restricted to a Th1 phenotype, as is the case with other intracellular pathogens *(12)*.

L. monocytogenes as a Vector for Tumor Antigens

In the present decade, there has been an emerging consensus that T cells, and especially class I-restricted $CD8^+$ T cells, are the critical mediators of an effective antitumor response *(13)*. Unlike antibody targets, T cell epitopes may be cytoplasmically located, and their epitopes consist of linear amino acid stretches. In the last few years, a number of tumor antigens as well as epitopes recognized by tumor-specific T cells have been characterized *(13)*. Consequently, current cancer vaccination strategies are primarily directed toward raising strong tumor-specific T cell responses and introducing these identified T cell antigens to the immune system in such a way as to prime an immune response that will be sufficient to eliminate tumor metastases and residual tumor mass *(14,15)*. A serious problem, however, in mounting an immune response to antigens expressed by tumor cells is that they are often

poorly immunogenic because of their strong homology to self-proteins. In addition, they will have been initially presented to the immune system in the context of tumor cells that are, for the most part, poor antigen-presenting cells likely to induce tolerance to the antigen rather than an active T cell response.

The ability of *L. monocytogenes* to stimulate strong cell-mediated immunity suggests that this bacterium may be an ideal vaccine vector to introduce poorly immunogenic tumor-specific antigens to the MHC class I and class II antigen presentation pathways in professional antigen-presenting cells. To test the efficacy of *L. monocytogenes* as a cancer vaccine, we have used a model tumor system that utilized influenza nucleoprotein as a model tumor antigen *(16,17)*. A recombinant *L. monocytogenes* vaccine strain (Lm-NP) that expresses NP from influenza strain A/PR8/34 was used. Lm-NP had previously been shown to present the K^d restricted NP epitope in vitro and induce NP-specific CTL in vivo *(18)*. To provide transplantable tumors expressing NP as a model tumor antigen, the highly tumorigenic, class I^+/II^- tumor cells CT26 and RENCA were transduced with the NP gene. We found that the therapeutic potential of this vaccine vector to limit tumor growth *(16)* was impressive. Regression of macroscopic tumors could be demonstrated for both tumor types *(16,17)*. The importance of T cells in the antitumor immunity was confirmed by histological analysis of the tumors and by in vivo depletion of either the $CD8^+$ or $CD4^+$ T cell subset. Both subsets clearly played a role in antitumor immunity despite the fact that both tumors are MHC class I^+/II^-, and class II expression on CT26 is not inducible. Thus, $CD8^+$ cells might well have been expected to be the sole effector mechanism operating in the tumor environment. Nevertheless, a consideration of the multifaceted way in which *L. monocytogenes* stimulates immunity may provide an explanation for these findings.

The frequent failure of the immune system to respond to tumor antigens and the subsequent outgrowth of transformed cells in cancer has been attributed to inadequacies of tumor cells in presenting antigens *(13,14)* and to tolerance mechanisms acting on tumor-specific T cells. This deficiency has been remedied by transfecting the tumor cells with certain cytokine genes, such as IFNγ, IL2, IL3, IL4, IL6, IL7, GM-CSF *(13,14,19–21)* or with costimulatory ligands *(12,22,23)* which either activate or bypass the requirement for T cell help in mounting a protective tumor-specific $CD8^+$ CTL response. Accordingly, the Th1 type cytokine profile induced by *L. monocytogenes* that we have described may play an important role in the efficacy of this vaccine vector. Indeed, we have demonstrated the presence of mRNA from a wide array of Th1-type cytokines in regressing tumors explanted from vaccinated animals and shown that depletion of the Th1-type cytokines IL12, IFNγ, and TNFα can abrogate the ability of *L. monocytogenes* to protect against tumor challenge in the NP model system (Pan et al., unpublished observations). The induction by Th1 cytokines of cell-surface molecules important in T cell activation is a considerable advantage in an anticancer vaccine. Thus, *L. monocytogenes* may be an ideal vehicle not only for presentation of tumor antigens to tumor-specific T cells, but also to provide the correct milieu to enhance the efficacy of these effector cells.

Our current studies are aimed at dissecting the mechanism by which *L. monocytogenes* causes the regression of established tumors in an antigen-specific manner. We are examining the correlates for immunity, including the type of lymphocytes and cytokines that are required for antitumor immunity, and addressing the possibility that the cell-surface phenotype of the tumor cells may change in response to the local environment provided by infiltrat-

ing Th1 $CD4^+$ and $CD8^+$ cells. We are also expanding our studies to more natural models of mouse and human tumors. It could be argued that influenza nucleoprotein is too immunogenic to be an appropriate model tumor antigen. In fact, the expression of NP in Renca and CT26 does not appear to enhance their immunogenicity or decrease their intrinsic tumorogenicity, and the minimal tumoricidal doses for Renca-NP and CT26-NP in BALB/c mice are identical to the parent lines (5×10^3/mouse). Furthermore, animals in which Renca-NP and CT26-NP are growing do not have functionally detectable NP-specific CTL. Thus, when expressed in the tumors, NP does appear to behave indistinguishably from an endogenous tumor antigen. However, to answer this criticism, we are currently expanding our studies to endogenous mouse tumor antigens, such as P1A, in the mouse mastocytoma P815 *(13)*, the murine leukemia retroviral product gp70 that is endogenously expressed by CT-26 *(24)*, and tissue-specific antigens in the melanin pathway such as tyrosinase and tyrosinase-related proteins, that are expressed by murine melanomas *(25,26)*. We are also investigating the potential of the *L. monocytogenes* vector as a vaccine against breast cancer. We are using the HER-2/neu transgenic mouse in which the oncogene is expressed in breast tissue to test the ability of our vaccine approach against spontaneously arising breast tumors in this animal model.

Our long-term studies, of course, are aimed at using *L. monocytogenes* as a cancer immunotherapeutic. To that end, we have recently constructed (Zubair et al., unpublished observations) two potential human cancer vaccine vectors that secrete the antigens E6 and E7 from human papilloma virus strain 16 the major causative agent for human cervical cancer *(27)*. Ninety percent of human cervical tumors express HPV-16 E7 protein, which makes it an excellent candidate for tumor-antigen-specific immunotherapy delivered by *L. monocytogenes*.

L. monocytogenes as a Vector for Viral Antigens

Cellular immunity has been shown to play a significant role in controlling viral infections *(28,29)* and yet, there are many viral infections for which the human immune response is unable either to prevent or clear infection for many individuals. One of the most devastating of these is HIV. There is increasing evidence that retroviruses can induce both humoral and cellular immunity. Indeed, it is now several years since HIV-specific CTL were discovered in healthy seropositive patients *(30–32)*, and since then there have been many studies which have confirmed the presence of CTL in HIV^+ humans specific for *gag, pol, nef, tat,* and *env* gene products *(33,34)*. The role of $CD8^+$ CTL in the immune clearance of retroviruses is still not entirely clear, but there are a number of studies suggesting that CTL may play a major role in protection against HIV and its simian counterpart. For example, a correlation between low viral titers and the presence of gag-specific CTL in peripheral blood has been shown in SIV-infected macaques *(35)*. These studies have encouraged many investigators to focus on CTL immunogenicity as a prerequisite for anti-HIV vaccines.

In HIV infections, antiviral antibodies are produced, but their ability to control the spread of the virus is clearly ineffective. The primary antigens of intact virus exposed to the humoral immune system are the envelope glycoproteins. The mutation rate of the *env* gene, however, is very high, and thus, viral escape mutants emerge that evade the humoral immune response *(36,37)*. In addition, antibodies against the major envelope protein gp120 have been shown not only to be ineffective in protecting against viral infection, but also to be implicated in disease progression *(38)*.

A low rate of mutation in the genes that code for core proteins is a major advantage in vaccines aimed at enhancing the cellular arm of the immune response to viruses, such as influenza and HIV, which have mechanisms for avoiding humoral immune responses. In contrast to antibodies, CTL are often broadly crossreactive, because they are easily invoked by core proteins, which readily target the class-I restricted pathway of antigen recognition. The prospects for similar vaccines, aimed at boosting the $CD8^+$ CTL response to HIV, are encouraging since a number of studies have shown that the CTL response to *gag (39–42)*, *nef (39,43–44)*, and *pol (45,46)* gene products are restricted to conserved sequences in these proteins from HIV-1 presented by a wide number of HLA class I alleles. Therefore, despite some reservations, there is good evidence that the $CD8^+$ class I-restricted arm of the cellular immune response may play a helpful role in reducing HIV disease progression in infected individuals, in addition to having a pivotal role in the design of a prophylactic anti-HIV vaccine.

An aspect of the immune response to HIV that has recently received a great deal of interest is the role of the balance of humoral and cellular immunity, as regulated by Th1 and Th2 $CD4^+$ subsets, in disease progression. Although still very controversial, it has been suggested that the humoral immune response, far from being beneficial or even ineffective in HIV infection, may actually be harmful, because it impairs the ability of the host to mount a protective cellular immune response *(47–49)*. This thesis is supported by other disease models. Thus, it has long been known *(50)* that either the humoral or cell-mediated arm predominates in the immune response to infectious agents, particularly intracellular pathogens, such as *Leishmania major* and *Mycobacterium leprae (12)*, resulting in either resistance or susceptibility to the disease. In support of the suggestion that this may also be the case for HIV infections are studies on HIV^+ individuals that indicated that there is a decline in T cell proliferation and IL2 production with a concomitant increase in B cell activity as AIDS symptoms progress *(49,51,52)*.

As we have discussed above, the $CD4^+$ T cell response in *L. monocytogenes* infections is predisposed to a Th1 response. In addition, we have shown that the intracytosolic location of this bacterium effectively targets recombinant proteins to the endogenous antigen-processing pathway. Thus, a recombinant *L. monocytogenes* based HIV vaccine may satisfy many of the parameters for HIV immunity that are not induced by HIV infection itself. To test the ability of recombinant *L. monocytogenes* to induce anti-HIV CTL, in collaboration with Frankel, we have constructed mutants stably expressing and secreting the gag protein of HIV type 1 *(53)* and *gag*, *nef*, and *env* of SIV (Yao et al., unpublished). Consistent with our findings in other antigen systems, strong $CD8^+$ T cell-mediated cytolytic responses were detected in mice immunized with *L. monocytogenes* that expresses gag *(53)*.

A further advantage to using *L. monocytogenes* as a vaccine vector is that it naturally infects through the oral route, and we have shown that it can effectively be administered as an oral vaccine for the generation of cell-mediated immunity against a recombinant antigen *(2,17)*. In the case of HIV infections, oral immunization could result in the induction of mucosal immune responses at the site of infection. We have shown that vaccination with recombinant *L. monocytogenes* can provide CTL responses effective against viral infection at the mucosal surface of the lungs *(18)*. Mice immunized with *L. monocytogenes* mutants expressing either a full-length influenza virus nucleoprotein or a construct that expressed only the MHC class I K^d binding epitope were subsequently infected intranasally with influenza virus. Pulmonary viral

titers were substantially reduced in immunized mice, relative to naive mice or mice infected with wild-type *L. monocytogenes (17)*.

We are currently examining the nature of the mucosal immune response to SIV antigens delivered by *L. monocytogenes*. Although strong $CD8^+$ and $CD4^+$ T cell responses are to be predicted, it is not obvious that the weak humoral responses detected when Listeria is delivered by a parenteral route would also prevail in the gut. It is currently thought that the mucosal immune response to soluble antigen is predisposed to a Th2 response, presumably because of the requirement for Th2 cytokines in the secreted IgA response. Thus, it is of interest to determine if the same would be true of antigens delivered by an intracellular pathogen capable of inducing cytokines that drive the T cell response in the other direction. Given that *L. monocytogenes* is a facultative intracellular bacterium, it is very possible that the bacterium is extracellular in this particular environment and competent, therefore, to induce an IgA response secreted by Peyer's patch B cells and intestinal epithelial lymphocytes. It is particularly important to establish this if this bacterium is ever to be used as an oral recombinant vaccine vector for either neoplastic or infectious disease. However, a problem arises in distinguishing between specific IgA produced in response to listerial antigens and IgA that is naturally occurring in the gut owing to responses to commensal bacteria. Nevertheless, by using *L. monocytogenes*, which expresses foreign antigens that are chemically distinct from prokaryotic proteins, we can use these as reporter antigens to quantitate immune responses against protein secreted by Listeria.

Intervention in Autoimmunity

Induced to Self-Antigens

EAE is one of the best-studied examples of an experimentally induced T cell-mediated autoimmune disease. It is an inflammatory disease of the central nervous system (CNS), which can be induced by injection of myelin basic protein (MBP), a common component of the CNS, with complete Freund's adjuvant (CFA) *(54)*. This results in the stimulation of a population of $CD4^+$ T cells, which mediate EAE and can transfer the disease to naive recipients. EAE resembles multiple sclerosis (MS), a human demyelinating disease of unknown etiology, both in its clinical course and histopathology. In addition, the presence of T cells responsive to MBP in the peripheral blood of MS patients *(55,56)* adds credence to the study of EAE as an animal model to explore the immune regulation of human demyelinating disease.

It has been found that the T cells, which mediate EAE in both the mouse and rat models, use a limited number of TCR genes. It was initially observed that the T cells generated both by the mouse (specifically the B10.PL and Pl/J strains) and by the Lewis rat in response to the encephalitogenic determinants of MBP showed restricted usage in both the variable region genes (expressed in the germ line) and also in the junctional regions generated when the TCR genes are rearranged *(54)*. An interesting finding was that the same variable regions were used by the mouse and the rat, even though the antigens and the MHC molecules recognized were different and functionally noncrossreactive. In both mice and rats, $V_{\beta 8}$ was used in combination with $V_{\alpha 2}$ or $V_{\alpha 4}$. This has led to the concept, called the V-region disease hypothesis *(57)*, which states that a specific $V_\alpha V_\beta$ combination, independent of its associated antigen MHC, is the cause of autoimmune disease.

Immunization with MBP-specific T cells, attenuated by irradiation, have been shown to prevent the induction of EAE in rats subsequently immunized with MBP and CFA *(58)*. Monoclonal antibodies (MAbs) directed

against the specific TCR variable regions can block expression of EAE and suppress ongoing disease *(59–61)*. Other studies have shown that immunization with TCR peptides corresponding to the Vα- and Vβ-chains can suppress EAE *(62–65)*, although TCR peptide immunization can in some cases lead to enhancement of disease *(66)*. The presumed mechanism of action of these therapies is to block the TCR on T cells responsible for disease with MAbs, or to induce a cellular immune response directed toward peptides derived from the TCR bound to MHC molecules on the encephalitogenic T cells.

Although it has been well established that $CD4^+$ T cells mediate EAE in the mouse and rat, the precise phenotype of the effector cells in the anti-idiotypic immune response to TCR determinants is as yet unknown, and in the rat, both $CD4^+$ and $CD8^+$ T cells have been implicated. In the mouse model, there is growing evidence that $CD8^+$ T cells are the major regulatory cell for EAE, and such cells have been strongly implicated in TCR peptide-induced unresponsiveness *(67)*. For example, mice depleted of $CD8^+$ cells by antibody-mediated clearance *(68)* or by "knockout" of the CD8 gene *(69)* showed less resistance to relapsing episodes of the disease. In addition, although TCR peptide-specific $CD4^+$ T cells have been isolated from mice recovering from MBP-induced EAE, they are thought to regulate the disease indirectly via a $CD8^+$ T cell mediator *(70)*. Given that mouse T cells, unlike their rat counterparts, do not express class II MHC, it appears unlikely that encephalitogenic $CD4^+$ T cells could be the cognate target of $CD4^+$-regulatory T cells in the mouse model. Our studies, therefore, concentrate on determining the presence of $CD8^+$-regulatory T cells against MBP-reactive T cells in the peripheral mouse repertoire, and what role they may have in regulation of EAE or in maintenance of tolerance in general in the mouse. In addition to providing a basis for possible autoimmune therapy, we believe our studies also address the whole issue of immune regulation and/or suppression in active cellular immune responses.

If indeed class I-restricted CD8+ cells play a role in immune regulation, then the question arises concerning whether current approaches to inducing immunoregulatory cells by immunizing with TCR peptides are the most appropriate. The T cell response to priming by soluble protein and peptide antigens almost always results in presentation of the antigens via the exogeneous cellular pathway and the priming of class II-restricted T cells. To induce class I-restricted T cells, antigenic peptides must usually be derived from intracellular processing of newly synthesized proteins within the cell cytoplasm, the so-called endogenous pathway *(71)*. This arm of the immune response has clearly evolved to deal with cytoplasmic infections by viral and bacterial intracellular parasites, which suggests that it may be best targeted by vectors of this type.

For a number of years, recombinant vaccinia virus has been the gold standard to study MHC class I-restricted T cell responses *(72)*. The use of vaccinia as a vector for the high level expression of foreign genes has become widespread, and the protocols are largely routine. Because homologous DNA recombination occurs during the normal replication of pox viruses, foreign DNA is easily inserted into the viral genome *(73,74)*. We have thus used vaccinia virus as a gene vector to target the Vβ TCR genes to the cellular arm of the immune response. We have made two constructs, one expressed the Vβ8.2 gene (VacVβ8.2) from the conalbumin specific T cell hybridoma D10 and the other expressed the Vβ3 gene (VacVβ3)from the pigeon cytochrome-c-specific T cell hybridoma 2B4 *(75)*.

To determine whether the VacVβ8.2 construct could regulate EAE, B10.PL or PL/J

mice were injected iv with 10^8 PFU of VacVβ3 or VacVβ8.2. Ten days later, EAE was induced, and mice were then examined daily for clinical signs. The mean clinical score after 30 d of antigenic challenge was 1.0 for the PL/J mice and 1.1 for B10.PL mice that had received VacVβ8.2, whereas the mice that received either VacVβ3 or no vaccine had mean clinical scores of 4.0–4.2. The profound alleviation of disease correlated with a reduction in the MBP proliferative response in mice vaccinated with VacVβ8.2, but not in mice that received VacVβ3. These proliferative responses could be reversed by the addition of IL2, suggesting that the vaccine had induced anergy in the antigen-specific T cells.

Recombinant vaccinia viruses have been used extensively to study the MHC class I-restricted CTL response to foreign antigens *(72)*. Thus, a possible mechanism for the concomitant reduction of MBP-reactive T cells and EAE in VVβ8.2-immunized mice could be the simple elimination of Vβ8.2 TCR bearing cells from the periphery of immunized mice by CTL killing. In order to compare the number of $CD4^+$ $Vβ8.2^+$ lymph node T cells from VVβ8.2-immunized mice with those immunized with VVβ3 or with naive mice, two color fluorescent-activated cell sorting (FACS) analysis was performed. The percentages of $CD4^+$ $Vβ8.2^+$ T cells were not significantly altered among the VVβ3, VVβ8.2, and naive groups of mice. However, it should be noted that the number of MBP-responsive cells in naive mice is so infrequent that their expansion by in vivo priming with MBP also does not alter the total number of $Vβ8.2^+$ T cells as measured by FACS analysis. Thus, it is possible that even a reduction of MBP-reactive T cells that is too small to be detected by FACS analysis could have an impact on the immune responsiveness of these animals to EAE.

To test this hypothesis, we used a mouse model that was transgenic for the TCR of AD10, a pCyt c-specific T cell, isolated from the $H\text{-}2^k$ mouse, whose TCR uses Vβ3Vα11 *(76)*. First ,we verified that normal $H\text{-}2^k$ mice immunized with VVβ3, but not VVβ8.2 showed diminished pCyt c proliferative responses. The reduction in responsiveness was very similar to the effects we see with MBP in the H-2u mice immunized with VVβ8.2 (Eidelman, et al., unpublished observations). Then T cells from the AD10 TCR transgenic $H\text{-}2^k$ mouse were adoptively transferred into syngeneic mice that had received VacVβ3, VacVβ8.2, or no vaccine, and the animals were subsequently immunized with pcyt c. The $Vβ3^+Vα11^+$ (AD10) pCyt c-responsive T cells were significantly reduced in the VacVβ3-immunized animals compared to the control group as determined by FACS analysis of draining lymph nodes. However, the $Vβ3^+Vα11^-$ population, which would not be activated by pCyt c, remained at similar levels. This indicates that immunization by VVβ3 appears to be influencing activated T cells only. We are currently verifying this finding in the MBP/Vβ8.2 model by repeating these experiments using a transgenic mouse constructed from a $Vβ8.2^+$ MBP-reactive encephalitogenic T cell line.

In other studies on the immune response to self-antigens, we are examining an unusual autoantigenic T cell response to the heme moiety of hemoproteins that we discovered some years ago *(77)*. This nonprotein, self-antigen is the prosthetic group of a number of hemoproteins, which are ubiquitous in most tissues, including the thymus. Therefore, it was of considerable interest to note that T cells freshly explanted from naive mice raised a potent proliferative response to heme in primary MLR, suggesting that heme reactive T cells occur at high frequency in the periphery *(77)*. T cells from mice of the $H\text{-}2^s$ haplotype (SJL, B10.S, and A.SW) exhibit a high responder phenotype when stimulated by heme in bulk primary assays, whereas mice of the $H\text{-}2^d$ haplo-

type (B10.D2 and BALB/c) are low responders *(77)*. Using limiting dilution analysis we found that the potent primary proliferative responses by T cells from H-2^s mice reflect a high frequency (0.26–0.45%) of heme-responsive T cells in the periphery *(78)*. This frequency is comparable to the frequency of alloresponsive T cells reported by others in primary mixed lymphocyte reactions (MLR). In keeping with their low responder phenotype in bulk primary assays heme-responsive T cells occur at about a 10-fold lower frequency in unprimed H-2^d mice (0.03%). Notably, even this figure is high by comparison to the frequency of T cells responding to a foreign protein antigen, horseshoe crab hemocyanin. Only 0.02% of T cells from SJL mice respond to HCHy even after expansion of this population by in vivo priming. In this respect, the response to heme differs not only from other self antigens for which T cell responses in healthy animals are generally detected only after considerable manipulation, but also from foreign proteins after in vivo expansion. We also have shown that T cells reactive to heme are similar to T cells reactive to peptide antigens in that they show strict MHC restriction and can use diverse V genes *(78)*. These features distinguish heme from superantigens and mitogens, which also stimulate potent primary MLR, but exhibit degenerate MHC restriction and, in the case of superantigens, restricted V gene usage. Using mass spectral analysis of naturally processed peptides bound to MHC class II molecules, we demonstrated that the set of peptides from cells pulsed with heme were quite different from those isolated from cells grown in the absence of heme. This suggests that T cells may be stimulated by heme via an alloreactive-like mechanism *(79)*. Currently, we are using a phage display library approach to determine if heme mediates this effect by binding to a set of peptides previously not presented to the immune system and facilitating their interaction with MHC class II.

The presence of such a high frequency of heme-reactive T cells in the peripheral lymphoid organs of mice and the absence of obvious pathology in these animals led us to investigate whether any heme-containing proteins could act as antigens to heme-reactive T cell clones *(79)*. Surprisingly, we found that the ability of the hemoprotein to stimulate T cell clones was species-specific. Thus, mammalian cytochromes-c were ineffective, but avian cytochromes were as potent as free heme. The poor antigenicity of mammalian proteins, however, could be reversed by denaturing the protein, suggesting that the native molecule is resistant to processing by murine antigen-presenting cells. Thus, heme appears to evade immune recognition in mice by sequestration as a cryptic epitope in some hemoproteins *(80)*. We are now investigating whether the population of heme-reactive T cells could be playing a role in the pathology of diseases in which heme levels are elevated or the normal pathway by which heme is degraded in the liver has failed to function.

Induced by Viral Infections

It is well known that many human autoimmune diseases are precipitated by viral infections *(80)*. Although the immune-mediated damage can often be traced to autoimmune T cells, it appears that viral infections can trigger these responses either by inducing inflammatory Th1-type cytokines that break tolerance to the auto-antigen or by a molecular mimicry mechanism, whereby a T cell response to a viral peptide sequence cross reacts with a homologous self-antigen *(81)*. Where viral infections are implicated in the initiation of autoimmune disease, this can obscure the exact etiology of the disease. This is the case with the human demyelinating disease MS. Although decades of research on the pathological, immunological, and biochemical aspects of the disease have taken place, the

etiology and pathogenesis of MS are still unclear. MS is an inflammatory, demyelinating disease in which the CNS lesions are heavily infiltrated by lymphocytes and macrophages. Inflammation of the CNS and the presence of increased MHC expression on resident cells in plaque areas *(82–84)* suggest autoimmune-mediated damage to the CNS. Epidemiological evidence, however, suggests there may be a viral infection involved in the etiology of MS *(85)*, and many attempts have been made to identify and/or isolate viruses from the CNS and blood of MS patients. However, despite reported successes over the years, in no case has a virus been unequivocally established as a causative agent of MS. It is quite possible, therefore, that the disease is fundamentally autoimmune in origin, but that a viral infection may trigger the response.

Although it is not clear that a virus is involved in the etiology of MS, infections of animals with viruses provide good model systems for the study of chronic demyelination in humans. Among these are intracranial infections with the coronavirus, mouse hepatitis virus (MHV). In these models, mice that survive acute encephalitis undergo chronic demyelinating disease, accompanied by inflammation. As in MS, there is uncertainty regarding the mechanism of demyelination or to what extent immune or autoimmune reactions contribute to it. In collaboration with the laboratory of Weiss, we are using coronavirus MHV strain A59 infection of mice *(86)* as a model system to study the role of the immune system in virally induced demyelination of the murine CNS.

The detection of viral particles in oligodendrocytes led to the theory that MHV-induced demyelination was directly owing to viral damage of these cells *(87)*. However, more recently it has been shown that immunosuppression, by irradiation of infected mice, prevented demyelination, despite a corresponding increase in viral titer *(88,89)*. This, together with the finding that demyelination was restored by the adoptive transfer of $Thy1^+$ splenocytes *(89)* suggested that T cells may be mediators of demyelination. In contrast to these studies, we have recently shown that $CD8^+$ T cell deficient $\beta 2M^{-/-}$ gene knockout mice undergo demyelination, indicating that $CD8^+$ T cells are not absolutely necessary for demyelination *(90)*. However, the frequency at which demyelination was observed in these animals was very low, a result which may be attributed to either a role for $CD8^+$ T cells in demyelination in normal mice, or an inefficient infection rate owing to the use of a low inoculating dose of wild-type MHV-A59 in $\beta 2M^{-/-}$ knockout mice (LD_{50} = 5 PFU). Although infection could reliably be established using C12, an attenuated variant of A59 (LD_{50} = 200 PFU in knockout mice), the question of the relative importance of $CD8^+$ T cells in demyelinating disease remained in some doubt, since C12 induces demyelinating lesions at very low frequency in both wild-type and $\beta 2M^{-/-}$ knockout mice.

In a recent study, we have attempted to resolve this question by carrying out in vivo depletions of $CD8^+$ T cells in normal C57BL/6 mice injected with 3000 PFU of wild-type MHV-A59 *(91)*. Similarly, we have extended our earlier study by addressing the role of $CD4^+$ T cells. We examined the effect of $CD4^+$ or $CD8^+$ T cell depletion at various time-points postinfection on the incidence of demyelination. An attempt was made to examine the role of $CD4^+$ or $CD8^+$ T cells in demyelination in the early stages of infection by MHV-A59 by commencing the depletion of each of these subsets on 5 dpi. Unfortunately, of six mice in each group infected in this experiment, all those depleted of $CD8^+$ cells and four depleted of $CD4^+$ cells died 15 dpi. This result was unsurprising given that viral titers are at a peak at 5 dpi and both $CD4^+$ and $CD8^+$ T cells are

known to be important in MHV clearance *(92,93)*. In order to reduce the risk of fatalities, this experiment was repeated, but the commencement of T cell depletions was delayed until 7 dpi, when viral titers were beginning to decline in thymectomized mice. Spinal cords were collected at 30 dpi and examined by toluidine blue staining for demyelinating lesions as described above. All mice examined in both control and $CD4^+$ or $CD8^+$ T cell-depleted groups contained demyelinating lesions.

We also performed the experiment beginning depletion of T cells at day 10 when viral titers are waning. This enabled us to also examine the effects of depleting both $CD4^+$ and $CD8^+$ T cells subsets, together, in addition to $CD4^+$ and $CD8^+$ T cells separately, without risk of persistent viral infection. Again, we saw no significant difference between any of the groups of mice with regard to demyelination at 30 dpi, compared to the control group, including the five mice in which both $CD4^+$ and $CD8^+$ T cells were depleted. These results suggest that neither $CD4^+$ nor $CD8^+$ T cells are the major effectors of MHV-induced demyelinating disease, but do not exclude a minor role of these T cells as effectors of demyelination.

Given our results, and those of Stohlman and colleagues *(88,89)*, which show that demyelination is reduced only if mice are immunosuppressed by irradiation at 6 dpi or earlier, with restoration of demyelination on adoptive transfer of $Thy1^+$ splenocytes, it seems probable that T cells may play a role in induction of demyelinating disease rather than as major effectors. It is possible that a component of early demyelination, prior to clearance of infectious virus, may be owing to direct virus-mediated lysis of oligodendrocytes. However, this seems unlikely to be responsible for the majority of the demyelination, which arises when infectious virus is no longer detected. The failure to suppress demyelination via irradiation at 7 dpi or later *(89)* would be consistent with a role for more radiation-resistant effectors, such as macrophages dependent on activation via T cells in the initial stages of the disease. In this regard, although $CD4^+$ T cells play a critical role in demyelination in experimental autoimmune encephalomyelitis and an as yet undefined role in MS *(94)*, macrophages are also conspicuous in the inflammatory lesions of these demyelinating diseases *(95,96)*. We are currently pursuing the source of CNS inflammation in MHV-A59-infected animals by examining the expression of cytokines in the CNS at various times of infection and post-infection during the demyelinating phase.

Concluding Remarks

It is obvious that the outcome of the immune response to a protein antigen, whether foreign or self, is governed by a complex series of in vivo events. These include the antigen-processing pathway accessible to the antigen, the level of costimulatory ligands on the antigen-presenting cell, the cytokine milieu in which the immune response develops, and the possible induction of immuno-regulatory cells. In this brief article, we have outlined some of the studies in these areas that engage and excite our laboratory. Our current approach is to use live recombinant vectors to manipulate these parameters. However, given that these organisms are precisely the stimuli to which the immune system has evolved, we believe that studying the immune response to the reporter antigens they express will shed light on fundamental immune mechanisms.

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