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<td>doi:10.1073/pnas.0611263104</td>
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Noninfectious entry of HIV-1 into peripheral and brain macrophages mediated by the mannose receptor

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Communicated by Robert C. Gallo, University of Maryland, Baltimore, MD, January 8, 2007 (received for review January 16, 2006)

Although protein receptors on the plasma membrane involved in the initial steps of productive HIV-1 infection have been well characterized, little is known about interactions between cellular carbohydrate receptors and HIV-1. Here, we report the involvement of a carbohydrate receptor, the macrophage mannose receptor (MR), and its role in supporting HIV-1 binding and entry. HIV-1 can enter the cytoplasm of human macrophages and microglia as well as murine macrophages by MR, although no subsequent viral replication was observed. Correspondingly, HIV-1 entry into COS-7 cells after induction of expression of MR by transfection with MR-cDNA did not demonstrate viral replication. Our studies suggest that whereas MR may serve as a binding and an entry site, the MR-mediated pathway does not lead to productive HIV-1 infection. In addition, we report that recombinant HIV-1 gp120 blocks MR-mediated phagocytosis in human and murine alveolar macrophages and microglial cells. Therefore, characterization of the HIV-1 noninfectious MR-mediated phagocytic pathway may foster advances in HIV-1 vaccine design and an improved understanding of HIV-1/AIDS pathogenesis and host defenses.

gp120 vaccines phagocytosis microglia

Over the last two decades, much progress has been made in identifying cellular protein receptors for HIV-1. For example, the first identified receptor for HIV-1, the CD4 molecule, was implicated in HIV-1 infection of T helper lymphocytes (Tropic virus), as well as monocytes/macrophages (M tropic virus) (1, 2). It was then demonstrated that other members of the chemokine receptor family could also function in combination with CD4 for HIV-1 fusion and entry into target cells (3–9). Specifically, Tropic HIV-1 utilizes the chemokine receptor CXCR4 as a coreceptor (8), whereas M tropic HIV-1 uses CCR5 and CCR3 (3–7, 9). However, we and others have shown that HIV-1 can infect several cell types that do not express CD4, including colonic epithelial brain-derived glial and neuronal cells (10–12). In these cells, although CD4-negative, the glycolipid GalCer can serve as an HIV-1 receptor (13).

The Env gene of HIV-1 encodes a glycoprotein precursor, gp160, that is cleaved intracellularly to form the mature gp120 and gp41 molecules (14–16). It has been well established that the selectivity of different strains of HIV-1 for specific cell types is regulated by interactions between the viral envelope and cellular receptors. Specifically, the CD4 and other chemokine coreceptors described above interact with the gp120 of the HIV-1 envelope. However, the protein portion of gp120 constitutes only half of its molecular weight. The remaining half of the total molecular weight is due to heavily glycosylated carbohydrate moieties (17–21). Indeed, studies have shown that endoglycosidase-treatment of gp120 to cleave internal polysaccharide linkages results in a protein of 60 kDa, the predicted size of an unglycosylated gp120 (17–21). The functional importance of these sugar residues was demonstrated by a study demonstrating that the exceptional density of HIV-1 envelope glycosylation prevented the binding of neutralizing anti-bodies but not receptor binding (22). Furthermore, two other studies have shown that high-mannose-type oligosaccharides of HIV-1 gp120 are recognized by the serum lectin, known as mannose-binding protein (23, 24). In addition, both macrophage MR and primary monocyte-derived macrophages have been shown to bind and facilitate HIV-1 transmission to T cells (25).

Interestingly, the pathogen Pneumocystis carinii (PC) also has a major surface glycoprotein with a molecular weight of ∼120 kDa (gpA/gp120) (26). The gp120 of PC is composed predominantly of high-mannose-type sugars, which bind to the cell surface receptor known as the mannose receptor (MR) (27). The MR is a 175-kDa transmembrane protein that has multiple domains (28, 29). Expressed on the surface of differentiated macrophages, Langerhans cells, and endothelial cells (19, 28), MR binds to carbohydrates of a wide array of infectious agents and mediates endocytosis, phagocytosis, and antigen presentation (19, 28, 30). Recently, MR has been shown to be an essential regulator of serum glycoprotein homeostasis (31).

Taking into consideration the similarity in gp120 carbohydrate composition of HIV-1 and PC, we explored the hypothesis that the gp120 of HIV-1 can interact with MR in a process analogous to the gp120 of PC. We sought to determine whether the binding of HIV-1 gp120 to MR of macrophages will lead to a noninfectious pathway for HIV-1. Our results demonstrate that the macrophage MR can serve as a receptor for HIV-1 by gp120 binding. Importantly, whereas HIV-1 did gain entry into the macrophages by the MR, no viral replication was observed. These studies suggest the role of MR as a potential site/receptor for the noninfectious entry of HIV-1. Our results also provide new insight into the mechanisms of an HIV-1 induced immune response in the absence of viral replication. Further examination of this pathway may have implications for HIV-1 vaccine design and may also lead to a more detailed understanding of host defense mechanisms and the dynamics of HIV-1/AIDS pathogenesis.

Results

Binding of HIV-1 gp120 to the Macrophage MR. To determine whether HIV-1 gp120 binds to the MR of macrophages, an MR-mediated phagocytosis assay of yeast particles using murine alveolar macro-

Author contributions: J.R.T., M.E., and J.D.B. designed research; J.R.T., R.R., R.M.M., F.D., and M.F.M. performed research; R.R. and F.D. contributed new reagents/analytic tools; J.R.T., R.R., R.M.M., F.D., M.F.M., M.E., and J.D.B. analyzed data; and J.R.T. and J.D.B. wrote the paper.

The authors declare no conflict of interest.

Freely available online through the PNAS open access option.

Abbreviations: PC, Pneumocystis carinii; MR, mannose receptor; gp120, recombinant HIV-1 gp120.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0611263104/DC1.

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phagocytosis model are analogous to what occurs in human cells, we obtained alveolar macrophages from a bronchoscopic alveolar lavage of a healthy individual with no known risk factors for HIV-1 infection (confirmed HIV-1 seronegative by ELISA). As shown in Fig. 1C, human alveolar macrophages also ingested yeast particles by phagocytosis. This phagocytic pathway was also MR-mediated, as determined by mannan-blocking (Fig. 1C Inset). Importantly, incubation of human alveolar macrophages in the presence of rgp120 blocked the intake of yeast particles (Fig. 1D). As with the murine model, we blocked phagocytosis using different concentrations of rgp120. For example, treatment of the human alveolar macrophages with 200 μg/ml rgp120 blocked the phagocytosis of yeast by ~80%. With 333 μg/ml rgp120, blockage was increased to 95% (see Fig. 1F).

Expression of MR in Human Microglial Cells and Binding of HIV-1 gp120. Microglia are important target cells during the early stages of HIV-1 infection of the central nervous system. Moreover, several protein receptors for HIV-1 have been identified on microglial cells including CD4, CCR5, and CCR3 (9). Although the MR is expressed on the surface of rat and mouse microglial cells (33–35), the expression of MR in human microglial cells has not been well characterized. Because of the importance of this cell type in HIV-1 pathogenesis such as AIDS dementia, it would be of interest to determine what carbohydrate receptors are present on these cells that might also bind HIV-1.

First, to determine whether MR is expressed on human microglia, we isolated microglial cells from the brain tissue of patients that underwent brain surgery for intractable temporal lobe epilepsy [supporting information (SI) Fig. 6A]. The sorted CD11b- (Mo1-FTIC) positive microglial cells (>98%) were resuspended in RPMI culture media (SI Fig. 6B). After 5 days in culture, microglial cells were collected, incubated with a polyclonal antibody against the MR, and analyzed by using flow cytometry. Approximately 80% of the microglial cells expressed MR on the cell surface (SI Fig. 6C). To determine the cellular distribution of the MR on human microglia, the cells were indirectly immunostained with fluorescent anti-MR and analyzed by laser confocal microscopy. Imaging of the immunolabeled cells revealed MR on the cell surface and in the cytoplasm (SI Fig. 6C).

Once MR was demonstrated on microglial cells, we examined whether HIV-1 gp120 could bind to MR by using MR-mediated phagocytosis assays of yeast particles, as described above, using murine and human alveolar macrophages. Phagocytosis of yeast particles was >95% inhibited with 333 μg/ml rgp120 (SI Fig. 6D). As a positive control for the MR-mediated pathway, 50 mg/ml mannan was used to block microglia phagocytosis in a competitive assay. As with alveolar macrophages, phagocytosis by microglial cells was more effectively blocked with rgp120 than mannan. The MR-mediated phagocytosis experiments were repeated twice, each yielding similar results. These data demonstrate not only the presence of MR on cultured human microglial cells but also the role of this receptor in the phagocytosis of yeast particles. We also found that rgp120 blocks phagocytosis by microglia. These data together suggest that gp120 of HIV-1 binds to the MR of macrophages and microglial cells, resulting in the blocking of phagocytosis of yeast via the MR.

Phagocytosis of PC Blocked by rgp120. In another approach to demonstrate HIV-1 gp120 utilization of MR, we performed a MR-mediated phagocytosis assay using FITC-labeled PC (FITC-PC), as described (27). Using confocal scanning microscopy, macrophages exhibited phagocytosis of the FITC-PC, as shown in Fig. 2A–C. When 50 mg/ml mannan was added to the media before FITC-PC exposure, phagocytosis was inhibited (see Fig. 2D–F). The addition of 333 μg/ml rgp120 to the macrophage cultures before FITC-PC exposure also resulted in the inhibition of phagocytosis (Fig. 2G–I). Additionally, when polyclonal antibodies to PC

Fig. 1. Binding of HIV-1 gp120 to the macrophage MR. (A) Phagocytosis of yeast particles (light purple) by the murine alveolar macrophages by MR. Inset is a positive control using mannan for blocking MR phagocytosis. The murine alveolar macrophages blocked with mannan did not phagocytize yeast particles. (B) Recombinant HIV-1 gp120 (rgp120) blocked the intake of yeast particles into murine alveolar macrophages by MR. (C) Phagocytosis of yeast particles by human alveolar macrophages. Inset shows phagocytotic blocking with yeast mannan. (D) rgp120 blocked the MR-mediated phagocytosis of yeast particles by human alveolar macrophages. (E) Phagocytic index using different concentrations of mannan and rgp120 with murine alveolar macrophages. (F) Phagocytic index using different concentrations of rgp120 in human alveolar macrophages.
were incubated with FITC-PC before exposure to macrophages, the FITC-PC were not taken up by the cells (Fig. 2 J–L). Experiments were repeated twice, each yielding similar results. These data suggest that gp120 of HIV-1 blocks MR-mediated binding and phagocytosis of PC by macrophages.

Role of MR in HIV-1 Infectivity. Having established that HIV-1 gp120 binds to the MR of macrophages and microglia, the functional consequences of HIV-1 binding to this receptor were studied. To select an HIV-1 viral strain that readily infects macrophages, we exposed HIV-1HIV, HIV-1ADA, HIV-1RFL, HIV-1MN, and HIV-1Bal isolates to human macrophage cultures. Over a 12-day period, all HIV-1 isolates produced significant infection in the macrophages, especially the HIV-1Bal and HIV-1RFL strains (data not shown). As a result, the macrophage-tropic HIV-1Bal strain was chosen for further experiments of infectivity (36).

To determine whether productive infection of macrophages/microglia by HIV-1Bal requires both MR and CD4 binding, blocking experiments were performed by using mannan and antibody against the gp120-binding site on the CD4 receptor (OKT4A). A dilution of 30 mg/ml mannan and 30 μg/ml of OKT4A was incubated with human alveolar macrophages (Fig. 3A), peripheral monocytes/macrophages (Fig. 3B), and microglia (Fig. 3C) for 1 h at 37°C. These pretreated macrophages and microglia were then exposed to the HIV-1Bal strain (2 × 10^6 TCID50). As shown in Fig. 3 A–C, OKT4A, which binds the gp120 binding site on CD4, blocked HIV-1 infection in peripheral and alveolar macrophages, as well as microglia. Interestingly, although MR was open for phagocytic entry of HIV-1 into these macrophages and microglia, no observable infection occurred. However, when 30 mg/ml mannan in the absence of OKT4A was incubated with parallel cultures of macrophages and microglia, productive HIV-1 infection was observed. When cells were treated with mannan and OKT4A together, as shown in Fig. 4 A–C, no viral replication was observed. Control experiments were performed by using 30 μg/ml bovine albumin, with no observable inhibition of viral replication.

Exposure of human macrophages to a dilution of 50 mg/ml mannan for 8 h resulted in the development of observable cytopathic effect (data not shown). Therefore, the experimental condition chosen for the blocking experiments was set at 30 mg/ml mannan. The blocking ability of several mannan dilutions extending downward from the 30 mg/ml were tested (30, 20, 10, 5, 1, 0.5, and 0.1 mg/ml) by incubation with human alveolar and peripheral macrophages for 1 h at 37°C. Infection was performed as mentioned above, and no blocking of infection in macrophages was observed with any of the mannan dilutions. These blocking experiments were repeated twice with similar results (data not shown). Taken together, the data indicate that mannan alone does not inhibit HIV-1 infection in alveolar macrophages, peripheral macrophages, or microglia.

Transfection of MR-cDNA in Cos-7 Cells: Consequences for HIV-1 Infectivity. As described (27), transfected Cos-7 cells induced to express MR are able to bind and ingest PC.
Here, we report that the macrophage MR also supports HIV-1 infection in Cos-7 cells transfected with MR-cDNA. Approximately 80% of Cos-7 cells were gag-positive (Fig. 4 D and E). Control experiments yielded only nonspecific binding in the nontransfected Cos-7 cells (Fig. 4 D inset and E inset), as well as transfected Cos-7 incubated with a control Ig isotype antibody or mock control (data not shown). Immunostaining results were reproduced in five separate experiments. Cells were also examined by electron microscopy. Interestingly, Fig. 4 G shows the presence of numerous dense virion-like particles in endosomal compartments of only the Cos-7 cells that had been transfected with MR-cDNA and exposed to HIV-1. In Cos-7 cells that were not transfected with MR-cDNA or subjected to mock viral exposure, no dense particles were observed (data not shown).

Collectively, these results indicate that HIV-1 gp120 interacts with MR, and that HIV-1 entry into cells by the MR-mediated pathway does not result in viral replication.

**Discussion**

Phagocytosis mediated by the MR is the first line of defense used by macrophages and antigen-presenting cells against numerous microorganisms that express mannose on their surface (28). Here, we report that the macrophage MR also supports HIV-1 binding and entry. Our studies suggest that although MR serves as a viral entry site, the MR-mediated pathway does not lead to a productive HIV-1 infection. This conclusion is consistent with the concept that the main function of the MR is related to phagocytosis. Abundant evidence demonstrates that HIV-1 gp120 interacts with particular cellular protein receptors leading to the selective infection of target cells. These protein/protein interactions among HIV-1 gp120, CD4, and chemokine receptors initiate the life cycle of HIV-1, especially plasma membrane fusion, resulting in the production of infectious particles from target cells. However, in the presence of MR, the glycosylated portion of gp120 appears to initiate phagocytic entry, which does not result in viral replication. In light of this evidence, we propose an expanded model of HIV-1 entry as outlined in Fig. 5. Two independent pathways may coexist in macrophages/microglia: pathway 1 is the currently accepted infectious route by
plasma membrane protein receptors; pathway 2 is a newly described, noninfectious, phagocytic route mediated by the MR.

This MR-mediated HIV uptake concept component is consistent with a previous model used to explain the relationship between mycobacterial lipoglycan lipoparinomannan (LAM) and monocyte-derived antigen-presenting cells (37–39). As with LAM, MR binds and initiates the uptake of HIV-1, which is then delivered to an endosome. In the endosome, HIV-1 is degraded, and viral antigens are presented by CD1b to elicit cell-mediated immunity. The initial steps of our model are supported by electron microscopy (Fig. 4G), which shows the presence of virion-like particles in endosomes in MR-cDNA transfected Cos-7 cells that were exposed to HIV-1, but not in Cos-7 cells without MR. Additionally, these cells showed significant cytoplasmic binding of anti-p24. Future studies will be required to establish the colocalization of HIV-1 products in endosomal compartments and their interaction with CD1b molecules.

It has been shown that dendritic cells and macrophages process HIV-1 epitopes presented through the exogenous MHC-I pathway (40). This processing leads to cytoplasmic T lymphocyte (CTL) activation in the absence of viral protein synthesis. Based on these findings and data presented here, we hypothesize that APC capture HIV-1 through MR-mediated phagocytosis, which may lead to CTL immune responses. Future studies will be critical in establishing whether this noninfectious entry pathway for HIV-1 can generate effective CTL responses that confer effective viral immunity.

In light of our findings, a vaccine design that exploits the noninfectious MR-mediated entry pathway may have advantages. In HIV-1 gp120, most N-linked glycosylation sites are found within conserved regions (40). These conserved sequences open the possibility of producing a broad-based vaccine backbone that would address all clades of HIV-1. A gp120 vaccine design that retains conserved glycosylation sites may encourage MR-mediated antigen uptake and CD1b antigen presentation, leading to enhanced cytoplasmic T lymphocyte responses. Additionally, gp120 modifications that interfere with protein receptor binding could be incorporated to circumvent infectious pathway uptake and/or eliminate molecular mimicry epitopes that may elicit deleterious autoimmune responses (41, 42). Vaccine strategies for pathogens such as alpha-, flav-, and influenza viruses that require low pH conditions (i.e., endosomes) for fusion may also benefit from approaches that use MR-mediated entry.

Our observations of interactions between HIV-1 and MR may also shed light on other aspects of HIV-1 pathogenesis. We know that the MR recognizes carbohydrates from a wide array of infectious agents, as well as mediating phagocytosis and antigen presentation. In individuals infected with HIV-1, the existence of glycosylated gp120 and its interaction with MR may adversely affect phagocytic properties, thus contributing to opportunistic infections. We do not know whether significant blocking occurs by gp120 in vivo, or whether such blocking could help explain the relationship between HIV-1 infection and increased susceptibility to PC, a pathogen which also binds to MR. However, a study has shown that each virion contains ~216 copies of gp120. In addition, gp120 can shed spontaneously with a half-life of 30 h in vitro at 37°C (43). Consequently, in an AIDS patient, gp120 levels have been measured at an average of 29 ng/ml (44).

Interestingly, Koziel et al. (45) have shown that MR down-regulation occurs in persons infected with HIV-1. They demonstrated that individuals infected with HIV-1 had a reduction in phagocytic activity and expression of MR on the surface of alveolar macrophages. Reduction in MR expression was significant in patients with no evidence of PC pneumonia. This suggests that something other than PC may be responsible for down-regulation of MR. Our studies of gp120 binding to alveolar macrophage MR may illustrate one mechanism by which the availability of MR is reduced in HIV-1-infected individuals.

It is also possible that the expression of MR on microglial cells is related to pathogenesis in HIV-1-associated dementia. Cells of microglial lineage constitute the majority of HIV-1-infected brain cells, which form multinucleated giant cells, the histopathological hallmark of productive HIV-1 infection in the brain (46). However, little is known about the carbohydrate receptors on human microglial cells that might also bind HIV-1. It has been reported (34, 35) that MR is expressed on microglial cells of rats and mice. Here we report that human microglial cells also express MR (SI Fig. 6) and display active phagocytosis of yeast particles, a classic diagnostic assay for the presence of MR. Confirmation that this phagocytosis is mediated by MR was obtained by specific inhibition with mannan and rggp120 of HIV-1. Our data also establish that this phagocytic pathway leads to nonproductive HIV-1 entry (Fig. 3C). Thus, detection of HIV-1 p24 using immunological studies on multinucleated giant cells from the brains of patients with HIV-1 encephalitis may not necessarily reflect productive HIV-1 infection.

Despite the fact that microglia are the most prominent immune cells in the central nervous system, astrocytes, which are susceptible to HIV-1 infection, may also serve as immune regulators in the brain. Several studies have shown that murine astrocytes express MR as regulated by antiinflammatory compounds (33, 35, 47). Liu et al. (48) described HIV-1 infection of astrocytes mediated by MRs using an expression cloning strategy. However, the kinetics of HIV-1 infection in primary human astrocytes is questionable, because it shows only one peak of minimal infection and a decreasing viral production in the following days. In contrast, their peripheral blood mononuclear cell control follows the ascending kinetics of HIV-1 infection. In addition, the presence of 8 μg of polybrene in tissue culture used during HIV-1 infection introduces an artifact that influences the infectivity results. A recent study has demonstrated rapid internalization of HIV-1 by MR, which leads to a decrease in viral longevity. However, macrophages were able to mediate transmission of bound HIV-1 to cocultured T cells. Inhibitors of macrophage MR binding blocked up to 80% of this transmission (25). Consequently, it is critical to elucidate the role that MR plays in astrocytes, especially for HIV-1 infection, antigen presentation and phagocytosis.

In toto, further examinations of the MR pathway may have implications for HIV-1 vaccine design and may lead to a more detailed understanding of host defense mechanisms and the dynamics of HIV-1/AIDS pathogenesis, particularly in the pulmonary and nervous systems.

Materials and Methods

Cells. A human CD4-positive tumor line, SupT1, was obtained from the AIDS Research and References Reagent Program (National Institutes of Health) and maintained as described (11, 12). Cos-7 cells were obtained from the American Type Culture Collection (Bethesda, MD) and were maintained as described (11, 12). Human alveolar macrophages were obtained by bronchoscopic alveolar lavage from consenting adults as described (36). Monocytes/macrophages were obtained from peripheral blood lymphocytes of a healthy HIV-1-negative blood donor. The peripheral blood mononuclear cells were purified through lymphocyte separation medium (Organon Teknika, Durham, NC), according to the manufacturer’s protocol. Monocytes were incubated for 24 h at 37°C, 5% CO2 in RPMI medium 1640 containing 20% heat-inactivated FBS and 50 units/ml1 penicillin and 50 μg/ml1 streptomycin.

Isolation of Microglia. Freshly derived (immediately ex vivo) human glial cells were obtained from brain tissue specimens of patients undergoing temporal lobe surgery for intractable epilepsy (see SI Materials and Methods). Cells were purified as described (49, 50).

Viruses and Viral Exposure. HTLV-IIB (HIV-1RbB), HIV-1ADA, HIV-189.6, HIV-1MN, HIV-1RFL, and HIV-1Bal isolates were obtained from the AIDS Research and References Reagent Program

Trujillo et al.  
PNAS  |  March 20, 2007  |  vol. 104  |  no. 12  |  5101  
MEDICAL SCIENCES
core protein, and IgG1 isotype control antibody (New England Nuclear, Waltham, MA). The polyclonal antibody to human MR was kindly provided by P. Stahl (Washington University, St. Louis, MO). The secondary antibody used for immunostaining was FITC-conjugated goat anti-rabbit (Sigma, St. Louis, MO).

**Flow Cytometry Analysis.** Microglia were incubated with FITC-conjugated monoclonal anti-Mo1, a-C3bi receptor (Beckman-Coulter, Fullerton, CA), at 1:100 in PBS with 10% human serum at 4°C for 30 min. Ig isotype and mock served as negative controls. After two washes and resuspension in PBS, anti-Mo1-labeled glia were sorted into the positive microglia population using a MoFlo flow cytometer (Cytomation, Troy, NY) by back gating the Mo1-positive cells to the forward angle scatter vs. 90° side scatter. This method consistently yielded 95–98% Mo1+ microglia.

**Phagocytosis.** Human and mouse alveolar macrophages and human microglia were used to ingest yeast particles and fluorescein-conjugated PC, as described (see SI Materials and Methods) (27, 45). The phagocytosis assay was performed by using the technique of Giamis et al. (32).

We thank A. Ezekowitz (Massachusetts General Hospital, Boston, MA) for the MR-cDNA, H. Koziel (Beth Israel Deaconess Medical Center, Boston, MA) for providing human alveolar macrophages; A. Harmnen (Trudeau Institute, Saranac Lake, NY) for providing PC; J. Farland, B. Meek, N. Goletiani, C. Kendrick, C. Ramirez, and F. Cedeno for technical assistance; P. Jensen, J. Hou, and C. VanWinkle for editing the manuscript; and D. Knipe, T. Fox, E. Major, J. Ancer, and E. B. Trujillo for comments on the manuscript. This work was supported by a grant from the National Heart, Lung, and Blood Institute of the National Institutes of Health. J.R.T. was partially supported by a Rubenstein Fellowship.