Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 7089–7093, September 1986
Neurobiology

Cellular localization of human immunodeficiency virus infection within the brains of acquired immune deficiency syndrome patients

(HIV/AIDS virus/encephalitis/*in situ* hybridization/immunocytochemistry)

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Communicated by Floyd E. Bloom, May 14, 1986

ABSTRACT **Dysfunction of the central nervous system (CNS) is a prominent feature of the acquired immune deficiency syndrome (AIDS). Many of these patients have a subacute encephalitis consistent with a viral infection of the CNS. We studied the brains of 12 AIDS patients using *in situ* hybridization to identify human immunodeficiency virus [HIV, referred to by others as human T-cell lymphotropic virus type III (HTLV-III), lymphadenopathy-associated virus (LAV), AIDS-associated retrovirus (ARV)] nucleic acid sequences and immunocytochemistry to identify viral and cellular proteins. Nine patients had significant HIV infection in the CNS. In all examined brains, the white matter was more severely involved than the grey matter. In most cases the infection was restricted to capillary endothelial cells, mononuclear inflammatory cells, and giant cells. In a single case with severe CNS involvement, a low-level infection was seen in some astrocytes and neurons. These results suggest that CNS dysfunction is due to indirect effects rather than neuronal or glial infection.**

A human retrovirus, called lymphadenopathy-associated virus (LAV) (1), human T-cell lymphotropic virus type III (HTLV-III) (2), or AIDS-associated retrovirus (ARV) (3), and recently renamed human immunodeficiency virus (HIV) (4), is the causative agent of acquired immune deficiency syndrome (AIDS). A dominant feature of AIDS is the neuropsychiatric component with the attendant symptoms of generalized encephalopathy that occur at various times during the illness (5–11). These symptoms range from simple impaired concentration and mild memory loss to severe global cognitive dysfunction, which may be accompanied by motor disorders. Attributing this dementia to a specific agent is difficult, but various types of subacute encephalitis consistent with a viral infection of the central nervous system (CNS) have been seen in the brains of autopsied patients (5–12).

HIV DNA and RNA sequences appear in CNS tissue of individuals with AIDS encephalopathy at higher concentrations than in lymphoid tissues—i.e., lymph nodes or spleen (13). Further, restriction enzyme analysis has characterized the HIV in the CNS as both chromosomally integrated and unintegrated, the unintegrated form suggesting a replication intermediate for virus within the CNS (13). Recently, HIV was grown from cerebrospinal fluid and CNS tissues of 24/33 AIDS patients and 13/14 AIDS patients in another study, further implicating HIV as a CNS infective agent (14, 15).

However, the CNS cell(s) infected by HIV and the pathogenic mechanism by which the virus causes encephalopathy are unknown. To address these issues we used DNA probes and *in situ* hybridization in concert with immunohistochemical staining to determine which cell(s) of the CNS express HIV gene products. We found that HIV predominantly infects the mononuclear macrophage, giant cell, and capillary endothelial cell in the brains of AIDS patients with subacute encephalitis. The cellular distribution of HIV in these brains resembles that seen with visna virus infection of sheep, an analogous lentivirus, and suggests a pathogenic mechanism of viral-induced encephalopathy other than that of direct neuronal infection.

MATERIALS AND METHODS

Tissue Specimens. The brains from 12 AIDS patients with neuropathologic evidence of viral encephalitis and from 4 non-AIDS patients (who had died during the same time period) were removed and immersion-fixed in 20% buffered formalin (4–48 hr *post mortem*). After 7–10 days of fixation the brains were dissected, and representative sections from the frontal cortex, temporal cortex, occipital cortex, cerebellar cortex, and deep grey and white matter were embedded in paraffin. Sections 6–10 μm thick were attached to polylysine coated slides with 1% Elmers glue. For immunocytochemistry the sections were deparaffinized in three changes of 100% histoclear. After blocking endogenous peroxidase activity with 3% H_2O_2 in absolute methanol, sections were rehydrated in graded ethanol baths.

Immunostaining. Immunostaining was done as previously described (16). Rabbit antisera directed against neuron-specific enolase, S-100 protein (a marker of neurocrest cells), glial fibrillary acidic protein (a marker of astrocytes), α_1-antitrypsin (a marker of leukocytes), and common leukocyte antigen (T-200) were diluted in phosphate-buffered saline (PBS) according to commercial protocols (Dako, Santa Barbara, CA). Rat antisera to human macrophages (Mac-1 and Leu-M2) was obtained from Hybritech (La Jolla, CA) and Becton Dickinson, respectively. Rabbit polyclonal antiserum to HIV *gag*-encoded proteins were obtained from V. Kalyanaraman (Division of Viral Diseases, Centers for Disease Control, Atlanta, GA). These antibodies recognized HIV *gag* p17/18 and p24. After incubation in the primary antiserum overnight at 4°C, sections were extensively washed and then incubated with biotinylated secondary antibodies (goat anti-rabbit, anti-mouse, or anti-rat immunoglobulins from Tago) at 23°C for 1 hr. Sections were again washed before incubation for 30 min in either an avidin-biotin-horseradish peroxidase (Dako) or an avidin-alkaline phosphatase complex (Miles Laboratories, Naperville, IL). Reactions were developed with either aminoethylcarbazole or fast blue substrates (16). For double-label immunocytochemistry sections were stained for cellular antigens with the fast blue substrate as described, and then a second round

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Abbreviation: CNS, central nervous system.

of antibody staining was performed using the anti-HIV antisera, followed by a nonbiotinylated goat anti-rabbit immunoglobulin and rabbit antiperoxidase/peroxidase complex with the amino ethylcarbazole substrate.

***In Situ* Hybridization.** Techniques of viral probe preparation and *in situ* hybridization have been described (16). In brief, a 6.6-kilobase *Hin*dIII fragment of HIV was subcloned from the plasmid pBenn2 (provided by Malcolm Martin, National Institute of Allergy and Infectious Diseases, Bethesda, MD) into the *Hin*dIII site of pLW107 (provided by L. Whitton, Department of Immunology, Scripps Clinic and Research Foundation, La Jolla, CA) that contains SP6 and T7 promoters at opposite ends of the polylinker DNA (J. L. Whitton, personal communication). This recombinant product was nick-translated with deoxyadenosine 5′-(α-[^{35}S]thio)-triphosphate and deoxycytidine 5′-(α-[^{35}S]thio)triphosphate (650 Ci/mM, Amersham; 1 Ci = 37 GBq) according to the method of Rigby *et al.* (17). DNA polymerase I was purchased from New England Biolabs. Nick-translated DNA was separated from free nucleotides by passage through spin columns (Bethesda Research Laboratories) centrifuged for 2 min at 1200 × *g*. The specific activity of the probe was 1×10^8 cpm/μg of DNA. Similarly prepared probes to herpes simplex virus, cytomegalovirus, and the vector plasmid clone were used as controls. *In situ* hybridization was performed on paraffin-embedded tissues by using techniques similar to those described by Brahic and Haase (18). Deparaffinized sections were permeabilized with 1% Triton X-100 and proteinase K (10 μg/ml for 20 min at 37°C) and covered with hybridization mixture (36) [5× hybridization salts, 50% formamide, 5× Denhardt's solution (Denhardt's solution = 0.2% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin), 10% dextran sulfate, 500 μg of salmon sperm DNA/ml, 250 μg of bovine liver RNA/ml, 0.5 μg of ^{35}S-labeled probe/ml, 0.1% Triton X-100, 30 units of heparin/ml, and 10 mM dithiothreitol]. After heating to 100°C for 2 min, hybridization was continued for 18 hr at 40°C. Subsequently the coverslips were removed and the slides were extensively washed for 30 min in 2× standard saline citrate (SSC: 1× SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7) at 21°C, 30 min in 0.1× SSC at 21°C, 15 min in 0.1× SSC at 65°C, and 15 min in 0.1× SSC at 37°C. Finally, the slides were coated with Kodak NTB-2 (diluted 1:1 with 0.66 M ammonium acetate) and exposed for 4 days at room temperature. Development with Kodak D-19 was followed by counterstaining with hematoxylin for histologic examination.

RESULTS

Brain tissues from 9/12 AIDS patients with histologic evidence of viral encephalitis were found to contain HIV nucleic acid and proteins (Table 1 and Fig. 1) using *in situ* hybridization and immunocytochemistry. HIV *gag* proteins were prevalent in mononuclear inflammatory cells. These cells were considered macrophages on the basis of nuclear morphology, abundant foamy cytoplasm, and location in inflamed tissues (Fig. 1 *A–D*). The cell type was confirmed with double-label immunocytochemistry by using antisera for macrophage antigens (Mac-1) and for HIV (Fig. 1*D*). Furthermore, these cells hybridized with the HIV nucleic acid probe (Fig. 1 *E* and *F*). Such cells were found scattered throughout the CNS parenchyma but were more frequent in white than grey matter.

Giant cells were also infected with HIV. Some had the typical morphology of Langhans'-type giant cells, whereas others contained compact, centrally located nuclei (Fig. 1 *B* and *C*). However, the precise lineage of these cells was difficult to identify because they did not label with antibody to common leukocyte antigen, Mac-1 or Leu-M2. Further, they showed weak staining with antibody to α_1-antitrypsin. On rare occasions, these cells were labeled with antibody to glial fibrillary acidic protein; but this staining pattern was particulate and thus probably the result of phagocytosis. None of these cells stained with antibody to neuron-specific enolase or S-100 protein.

Capillary endothelial cells in tissues from non-AIDS patients (Table 1, patients 13–16) were difficult to identify in paraffin sections. In contrast, endothelial cells in brain tissues of AIDS patients were visible because of increased cytoplasmic volume. In the brains of 7/9 patients, staining with antiserum to HIV filled the cytoplasm of capillary endothelial cells (Fig. 1*G*), an observation that ruled out the possibility that the stained cells were inflammatory cells migrating through the vascular wall. This point was reemphasized by the appearance of positively staining endothelial cells in regions devoid of inflammatory cell infiltration. *In situ* hybridization with the HIV probe also localized viral nucleic acid to capillary endothelial cells (Fig. 1*H*) in grey and white matter. None of the brain tissues of non-AIDS patients hybridized with the HIV probe or stained with HIV antisera (Table 1). Nor did tissues from AIDS patients hybridize with radiolabeled control probes lacking the HIV insert (data not shown).

The immunostained antigen was restricted to small foci within the brains of 2/9 HIV-infected patients, while brains of the other seven patients exhibited severe involvement in most of the six areas sampled. In all cases, white matter was more severely affected than grey matter, but the inflammatory response was less than expected with a conventional viral CNS infection (i.e., cytomegalovirus, herpes simplex, measles) (19). In the most severely infected patient, 9, neurons and astrocytes stained weakly for HIV antigens.

DISCUSSION

HIV infection has been demonstrated in the brains of 9/12 AIDS patients who have histologic evidence of viral encephalitis. In eight of these patients viral nucleic acids and proteins were detected only in capillary endothelial cells, mononuclear inflammatory cells, and multinucleated giant cells. An additional infection of neurons and glia was seen in only one patient. This patient (Table 1, 9) had large amounts of viral antigen demonstrated by immunocytochemistry in many cell types. In all other patients, organic brain disease without significant neuronal infection was found. The detection limits of this assay have been determined to be 10–100 copies per cell (20). Thus, neuronal cells could carry a "latent" HIV infection that is nondetectable at our technical sensitivities. The data suggest, though, that endothelial cells and macrophages are the significant target cells and that CNS dysfunction does not require direct CNS infection.

That the macrophage is the most frequently involved cell during AIDS infection is an interesting observation. It is controversial whether microglia, the macrophages of the CNS, are primary CNS cells or represent monocytes that have migrated into the CNS (21). On the basis of analogy with all other CNS infections, however, the cells in perivascular cuffs are considered hematogeneous in origin. Given the low percentage of cells—$1/10^4$ (unpublished data) to $1/10^5$ (22) that carry HIV in the blood, the high percentage of infected macrophages in the CNS deserves comment. Progression of infection may be similar to that seen in visna virus infection *in vitro*, where viral expression and replication are dependent upon the maturity of infected cells. Initially, a few blood monocytes show low levels of visna RNA transcription, but stimulation and maturation into macrophages greatly amplifies viral replication (23). This observation has intriguing implications for HIV infection *in vivo*. Circulating mononuclear cells might harbor a latent infection but differentiate to a state supportive of HIV infection only after migration into

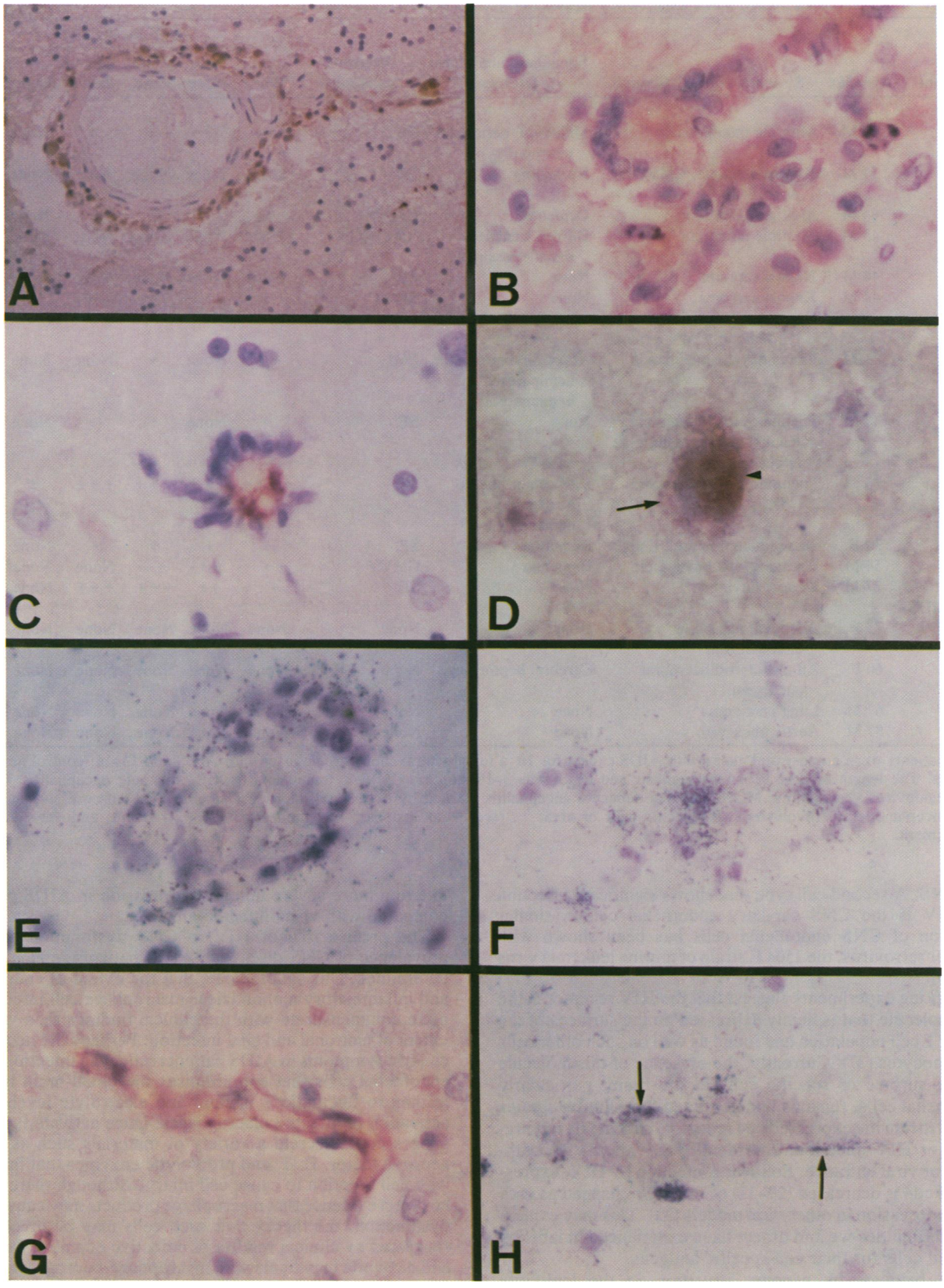

FIG. 1. Paraffin sections from brains of AIDS patients labeled by immunocytochemistry or by *in situ* hybridization. (*A*) Antisera to HIV stains inflammatory cells surrounding a blood vessel and cells invading the tissue. (×18.) (*B* and *C*) Antisera to HIV shows staining of mononuclear inflammatory cells and giant cells. (×45.) (*D*) Double-label immunocytochemistry showing that some macrophages label with both monoclonal antibody to Mac-1 antigen (arrow) and antisera to HIV (arrowhead). (×45.) (*E* and *F*) *In situ* hybridization with a ^{35}S labeled probe for HIV shows that perivascular mononuclear inflammatory cells and parenchymal macrophages hybridized with the HIV probe. (×50.) (*G*) Antisera to HIV shows infection of endothelial cells. (×45.) (*H*) *In situ* hybridization using a ^{35}S labeled probe for HIV shows that endothelial cells hybridize (arrows) with the viral probe (×45).

Table 1. Localization of human immunodeficiency viruses in the CNS of AIDS patients

Patient	Age/Sex	Systemic autopsy findings	Significant neurologic findings	Neuropathology	HIV localization					
					White	Grey	Monocyte	Endothelial	Neuron	Glia
AIDS										
1	39 M	Multiple infections	Multifocal peripheral neuropathy	SE	++	+	+	+	None	None
2	54 M	Kaposi sarcoma, CMV lung & adrenal	No history	SE	None	None	None	None	None	None
3	44 M	Multiple infections	Dementia	CMVV	++	+	++	++	None	None
4	43 M	Multiple infections	Diffuse weakness distal>proximal	Rare MGN	None	None	None	None	None	None
5	44 M	Kaposi sarcoma	None	SE	None	None	None	None	None	None
6	44 M	Kaposi sarcoma Cryptococcosis of lymphnode, CMV-adrenal	None	SE Cryptococcosis	+	+	+	+	None	None
7	31 M	Pneumocystis pneumonia	Ataxia, hyperreflexia, hemiparesis, hemianopsia	PML	+	None	+	None	None	None
8	40 M	Pneumocystis pneumonia, Kaposi sarcoma, multiple infections	None	SE	+	None	+	+	None	None
9	26 M	Pneumocystis pneumonia	Diffuse peripheral neuropathy, dementia	SE	++++	+++	+++	+++	±	+
10	30 F	Disseminated herpes simplex	Dementia	SE	+	+	+	+	None	None
11	54 M	Pneumocystis pneumonia	Dementia	CMVV	+++	++	++	None	None	None
12	27 M	Kaposi sarcoma	None	SE	++++	++	+++	+++	None	None
Non-AIDS										
13	73 F	Adenocarcinoma of endometrium	None	None	None	None	None	None	None	None
14	60 F	Atherosclerotic cerebral aneurysm	Cerebral hemorrhage	None	None	None	None	None	None	None
15	67 M	Lung carcinoma	None	None	None	None	None	None	None	None
16	87 M	Aortic aneurysm	None	None	None	None	None	None	None	None

All patients were homosexual males with AIDS except for 10, a female intravenous drug abuser with AIDS and 13–16, control non-AIDS patients. The length of time between diagnosis and death was 3–18 months. CMV, cytomegalovirus; SE, subacute encephalitis; CMVV, cytomegalovirus ventriculitis; MGN, minimal subacute encephalitis marked by occasional microglial nodule; PML, progressive multifocal leukoencephalopathy. Involvement of specific cells or area: ±, rare; +, occasional; ++, moderate; +++, frequent; and ++++, >50% involvement.

the CNS. A second cell type that shows significant infection by HIV is the CNS capillary endothelial cell; a similar infection of CNS endothelial cells has been shown with another retrovirus, the 1504 E strain of murine leukemia virus (24–26).

Blocking experiments suggest that the HIV receptor is the Cd4 molecule that is highly expressed on the surfaces of the helper T cell population and found as well on such other cells as monocytes (27). Currently, the presence of this molecule on the surface of specific cells in the brain, particularly endothelial cells, remains undetermined (28). Future studies should locate the expressed cd4 receptor, indicate its requirement for HIV infection, and identify any alternative pathways for viral entrance. Following infection by HIV, expression of cd4 is decreased (29–31) or might be changed as with HLA expression in other viral models (32). This may explain some difficulties we and others have experienced in labeling infected cells for their cell-specific antigens.

HIV belongs, by genome structure, in the lentivirus subfamily of retroviruses (33). The CNS infection by HIV demonstrated here shares several biologic features with CNS infection by visna virus. As observed with HIV (34), visna has a long (months to years) incubation period and involves the CNS. The HIV infection reaches deep white matter as does visna, but unlike visna viral infection the HIV infection does not elicit an intense inflammatory response. This may be due to a more severe immunosuppression in AIDS patients compared with visna-infected animals.

The precise role of HIV in CNS dysfunction and the prevalence of CNS disease in AIDS patients remains to be determined. Our data indicate that the extent of endothelial and inflammatory mononuclear macrophage cell infection is more commensurate with the clinical findings than was the extent of neuronal and glial infection. Perhaps the neuropsychiatric symptoms in AIDS patients reflect an infection of the endothelial cells that compromises the blood-brain barrier, leading to fluctuations in fluid and electrolyte levels or to activation of infected macrophages. Once activated, macrophages could secrete a variety of materials such as tumor necrosis factor, IL-1, and proteolytic enzymes that interfere with cell function to cause cell injury. Although there is still no direct evidence that macrophage products may cause CNS dysfunction, the theory that such cells may be involved is suggested by clinical reports of defective cognitive function and dementia in patients with macrophage-microglia involvement of the brain in the absence of a mass lesion (35). The CNS viral infection we describe for HIV has implications for therapy and prevention. Since neuronal infection is relatively insignificant, viral clearance of the brain parenchyma might prove an important research and clinical objective.

We thank Nancy Keating and Pat Burrola for technical assistance and Gay Schilling for secretarial support. This work was supported

in part by U. S. Public Health Service Grants NS-00928, AI-21640, NS-12428, AI-07007, and AG-04342. C.A.W. is the recipient of University of California Academic Senate Award RK211-M and Teacher Investigator Development Award NS-00928 from the National Institutes of Health. J.A.N. is the recipient of an American Cancer Society Junior Faculty Award (JFRA-115). This is Publication Number 4334-IMM from the Department of Immunology, Scripps Clinic and Research Foundation, La Jolla, CA.

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