

## Characterization of Cell Death Pathways in Human Immunodeficiency Virus-Associated Encephalitis

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**Human immunodeficiency virus (HIV)-associated dementia is a neurodegenerative syndrome characterized by cognitive decline, personality change, and motor deficits. HIV-associated encephalitis (HAE), the neuropathology responsible for HIV-associated dementia, involves the formation of multinucleated giant cells or syncytia. In this article we describe the apoptotic pathways activated in the brains of HAE-affected patients. Approximately 50% of multinuclear giant cells exhibited apoptotic DNA fragmentation as detected by the terminal dUTP nick-end labeling technique. In addition, the presence of syncytia in the frontal cortex of ~35% of HAE patients correlated with the number of cells expressing the HIV-1 protein p24. Histochemical and immunohistochemical analyses revealed that HAE-associated syncytia underwent apoptosis through a mitochondrial pathway previously delineated for HIV-1 envelope-elicited syncytia *in vitro*. We observed over-expression of the mammalian target of rapamycin (mTOR), a kinase that mediates activation of the pro-apoptotic transcription factor p53, and p53-dependent up-regulation of two effectors of mitochondrial apoptosis, namely the BH3-only proteins Puma and transglutaminase type 2 (TG2). Interestingly, although mTOR activation and Puma induction were observed in dying syncytia and neurons, I $\kappa$ B phosphorylation and TG2 up-regulation were only found in syncytia. These findings provide substantial new information on the cell death mechanisms that regulate HAE, suggesting an important pathogenetic role of syncytia in the disease. (*Am J Pathol* 2005, 167:695–704)**

Infection with the human immunodeficiency virus-1 (HIV-1) is associated with a progressive decrease in CD4 T-cell number and a consequent impairment in host immune defenses. It has been recently estimated that ~10% of HIV-infected patients develop HIV-associated dementia, a neurodegenerative syndrome characterized by cognitive decline, personality change, and motor deficits.<sup>1</sup> The neuropathology, particularly that associated with HIV-associated dementia, is characterized by a subacute HIV-associated encephalitis (HAE) with a variable degree of perivascular inflammation.<sup>2</sup> Mononuclear (microglial) cells are present throughout gray and white matter and tend to cluster in loose aggregates in association with occasional reactive astrocytes. A mild gliosis is found in the gray matter, both in the cortex and in the central gray structures. The white matter is also punctuated by small foci of active demyelination, usually with a paravascular or perivascular distribution. Reactive astrogliosis may extend into subcortical gray structures. In milder cases, the inflammatory changes consist only in a few perivascular lymphocytes and brown-pigmented macrophages. In more severe cases, the infiltrates consist of perivascular and parenchymal macrophages and multinucleated cells of macrophage origin. These infiltrates are also found in the white matter and in deep gray structures, especially the basal ganglia and thalamus, as well as in the brain stem.<sup>3–5</sup>

The pathogenetic mechanism(s) at the basis of HIV-induced neuronal injury is not fully elucidated.<sup>1,6,7</sup> HIV enters the central nervous system (CNS) early in the course of infection through infected macrophages and resides both in macrophages and microglia. Considering that neurons are not productively infected by HIV, it appears that the damage<sup>8–11</sup> affecting neurons is due to the action of toxic products (such as cytokines, chemokines, and endothelial adhesion molecules) released by in-

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**Table 1.** Characteristics of Enrolled Patients

|             | All (n = 43) | HAE with syncytia (Syn <sup>+</sup> ) (n = 6) | HAE without syncytia (Syn <sup>-</sup> ) (n = 11) | AIDS-related brain pathologies comprehensive of opportunistic infections and viral encephalitis (n = 26) |
|-------------|--------------|---|---|--|
| Age (years) |              |   |   |  |
| Median      | 38.8         | 37  | 35.5  | 40.6   |
| Range       | 30–55        | 30–50   | 24–48   | 30–55  |
| Sex         |              |   |   |  |
| Male        | 31           | 4   | 9   | 18   |
| Female      | 12           | 2   | 2   | 8  |

ected macrophage-microglial cells. However, it has also been proposed that HIV-1-encoded proteins (gp120, Vpr, and Tat) might participate in the neurodegenerative process.<sup>1,12–14</sup> In transgenic animals, gp120 (which is one of the proteins derived from the *env* gene of HIV-1) is neurotoxic and activates caspase-dependent apoptosis.<sup>15</sup> Accordingly, active caspase-3<sup>16</sup> and p53<sup>17</sup> have been detected in the brain of HAE patients.

One of the most important neuropathological alterations associated with HAE is the appearance of multinucleated giant cells or syncytia.<sup>1,18–20</sup> Syncytia are a common characteristic of HIV-infected tissues and are present in lymphoid tissues, in the brain, and in the spinal cord.<sup>19,21</sup> In the blood and lymph nodes, syncytia arise from the fusion between noninfected and HIV-infected cells through the binding of the HIV-1 envelope (Env) glycoprotein complex (gp120/gp41) to its receptor CD4 and a co-receptor of the chemokine receptor family.<sup>22,23</sup> HIV-1 strains that use CXCR4 as co-receptor are particularly efficient in eliciting syncytia.<sup>24–26</sup> The CXCR4 chemokine co-receptor is expressed in neurons, astrocytes, microglia, and endothelial cells in the brain.<sup>27–30</sup>

We have previously characterized the molecular pathway of apoptosis activated in syncytia both *in vitro* and in lymphoid tissues of HIV-1-infected patients.<sup>31–34</sup> Env-elicited syncytia manifest the up-regulation and nuclear translocation of mammalian target of rapamycin (mTOR) and mTOR-mediated phosphorylation of p53 on serine 15 (p53S15P), as well as the transcriptional activation of p53 correlating with its phosphorylation on serine 46 (p53S46P).<sup>31,32</sup> p53 then elicits the transcriptional up-regulation of the proapoptotic BH3-only protein Puma, which in turn mediates the insertion of Bax into mitochondrial membranes, thus causing the Bax-dependent mitochondrial release of cytochrome c with subsequent caspase activation and apoptosis.<sup>33</sup> In this system, another transcription factor, nuclear factor (NF)- $\kappa$ B, is activated after the phosphorylation of its inhibitor I $\kappa$ B, and cooperates in the process allowing mTOR to gain access to the nucleus (and hence to activate p53).<sup>33</sup> Based on these premises, we decided to explore the phenotype of brain syncytia in HAE. Here we report immunohistochemical evidence suggesting that HAE-associated syncytia die by an apoptotic pathway involving NF- $\kappa$ B, mTOR, p53, Puma, as well as another BH3-only protein, transglutaminase type II (TG2).

## Materials and Methods

### Patients

Autopsy samples of frontal cortex from 43 patients enrolled in prospective studies on HIV-related neuropathology were obtained from the Institute of Pathological Anatomy, Catholic University of Rome, School of Medicine. Seventeen cases were diagnosed as HIV-associated encephalitis (HAE) without superinfection by pathological and histological examination. The remaining samples presented additional opportunistic infections or CNS pathologies unrelated to HIV encephalitis (Table 1). All patients were not treated with highly active anti-retroviral therapy. Brain sections from three control patients were also included in the study. The autaptic samples were formalin-fixed at the time of autopsy and paraffin-embedded.

Macroscopically the most prevalent finding of HAE was pallor of the white matter, more severe in the central and periventricular white matter than in subcortical fibers. The hallmarks of HIV-associated encephalitis were: microglial nodule formation and multinucleated giant cells (syncytia); reactive astrogliosis, indicative of astrocyte activation; myelin pallor or the loss of myelin surrounding neuronal axons, indicating injured oligodendrocytes. Collectively these inflammatory and neuropathological findings based on extensive sampling of cerebral gray and white matter, basal ganglia, thalamus, brain stem, and cerebellum were independently evaluated by two pathologists (L.M.L. and V.A.).

### Immunohistochemical Analysis

Paraffin-embedded human frontal cortex sections (5  $\mu$ m thick) were deparaffinized, rehydrated, and subjected to high-temperature antigen retrieval in 10 mmol/L sodium citrate buffer, pH 6.0. Endogenous peroxidase activity was blocked by 3% H<sub>2</sub>O<sub>2</sub>. The primary antibodies used are: mouse RAFT1/FRAP (BD Transduction Laboratories, San Diego, CA) that label the factor mTOR, rabbit p70 S6 kinase (Thr421, Ser 424; Calbiochem, La Jolla, CA) that is the downstream target of mTOR, rabbit phospho-p53 (Ser 46), mouse phospho I $\kappa$ B- $\alpha$  (Ser 32/36; Cell Signaling Technologies, Beverly, MA) that is a marker for NF- $\kappa$ B activation, mouse Puma/Bbc3 (Upstate, Lake Placid, NY), mouse transglutaminase II Ab-1 (CUB 7402; Neo-

Markers, Fremont, CA), mouse human immunodeficiency virus p24, mouse neurofilament 200 kd (Novocastrol, Newcastle, UK), rabbit glial fibrillary acidic protein (DAKO, Carpinteria, CA).

The incubation with primary antibody was performed for 1 hour at room temperature and followed by a biotinylated goat anti-mouse or goat anti-rabbit IgG as secondary antibody. Then the incubation with a preformed horseradish peroxidase-conjugated streptavidin (Biogenex, San Ramon, CA) was performed. The immunoreaction product was revealed using aminoethylcarbazole or 3–3' diaminobenzidine as chromogenic substrates and 0.01% H<sub>2</sub>O<sub>2</sub> (Biogenex). As controls, we performed stainings with nonimmune isotype-matched control antibodies and by omitting the primary antibodies. Apoptotic cells were identified by terminal dUTP nick-end labeling (TUNEL) technique (*In Situ* Cell Death Detection kit; Roche Diagnostics, Penzberg, Germany). Sections were counterstained in Mayer's acid hemalum and analyzed.

#### Double-Labeling Protocol by Confocal Microscopy

Paraffin-embedded sections were deparaffinized, rehydrated, and treated with 0.2% Triton X-100 and 1% bovine serum albumin in phosphate-buffered saline. The incubations with primary antibodies were performed for 1 hour at room temperature and followed by the appropriate secondary fluorescent antibodies (Alexa Fluor; Molecular Probes, Eugene, OR) followed by TUNEL labeling fluorescein conjugated. The sections were analyzed with the Leica confocal microscope SP2.

#### Statistical Analysis

The brain sections of the 17 patients were semiquantitatively evaluated under code by three independent observers using a light microscope without the knowledge of either clinical or histological diagnosis. For each slide, a minimum of 10 fields was examined at  $\times 40$  magnification. To describe the histopathological brain alterations a semiquantitative evaluation scale was used based on the percentage of each parameter considered; tissue sections were scored as 0, if the inflammatory/degenerative changes involved an area comprised between 0% and 5% of the total section; 1 if 5 to 25%; 2 if 25 to 75%; and 3 if  $>75\%$ . To describe the degree of the p24 expression and TUNEL positivity on each specimen, a value comprised between 0 and 3 was assigned based on the percentage of positive cells present on the section as follows: 0: 0 to 5% of stained cells were present; 1: 5 to 25%; 2: 25 to 75%; and 3: 75 to 100%. The Mann-Whitney test for nonparametric data was used to compare medians, and the Pearson's correlation coefficient was used. Statistical significance was set at  $P < 0.05$ . The statistical analysis was performed by SPSS 11.0.1 for Windows (SPSS Science, Chicago, IL).

## Results

### The Presence of Dying Syncytia as an Index of HAE Severity

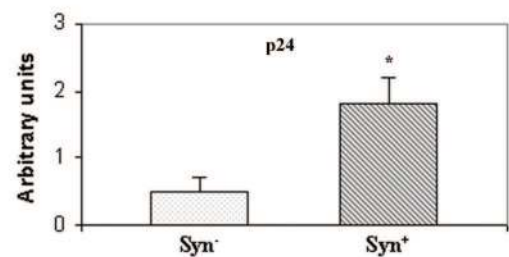
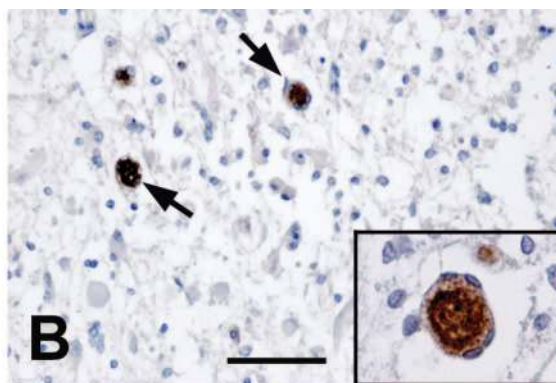
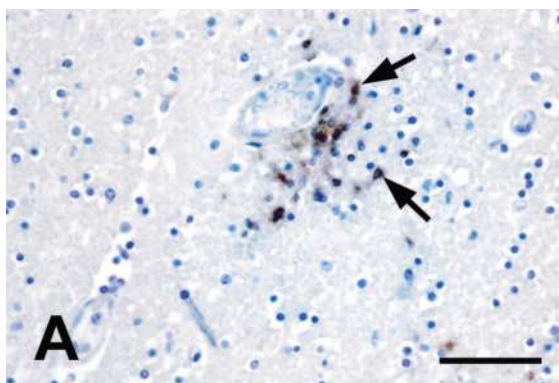
We analyzed autaptic human brain sections from 43 HIV-1-infected patients with different brain pathologies (Table 1) and 3 brains from healthy controls. We subsequently focused our investigation on 17 patients with confirmed HAE, without opportunistic infections or unrelated CNS pathologies. HAE brain sections exhibited perivascular infiltration (Figure 1A) associated with prominent astrogliosis, microglial nodules, and necrotic areas without the presence of syncytia (Syn<sup>-</sup>, Figure 1A) in 11 of 17 cases; whereas  $\sim 35\%$  of the patient samples with HAE were characterized by the presence of multinucleated giant cells/syncytia (Syn<sup>+</sup>, Figure 1B). The presence of HIV-1 in HAE brain sections was investigated by immunohistochemical staining with monoclonal antibodies specific for the HIV-1 p24 protein. p24 staining was observed in glial cells, as well as in the multinuclear giant cells (Figure 1, A and B). p24 staining was more pronounced in Syn<sup>+</sup> than in Syn<sup>-</sup> samples (Figure 1C): in fact, a significantly higher presence of p24 was measured ( $P = 0.015$ ) in Syn<sup>+</sup> sections, where the vast majority of syncytia themselves stained positive for p24 ( $90.25\% \pm 11.09$ , number of patients = 6) (Table 2). Moreover, a statistically significant correlation was found between the number of p24-positive cells and the percentage of syncytia in each specimen ( $R^2 = 0.846$ ,  $P = 0.0094$ ).

To study the fate of HAE-associated syncytia, we analyzed our samples by means of the TUNEL technique that detects apoptotic DNA fragmentation (Figure 1, E and F). With respect to Syn<sup>-</sup> patients, sections from Syn<sup>+</sup> patients tended to contain a higher frequency of TUNEL-positive cells. Even though this difference was not significant by statistical analysis ( $P > 0.05$ ) a high percentage of syncytia ( $48.9\% \pm 23$ , number of patients = 6) contained at least one TUNEL-positive nucleus (Figure 1G). It is noteworthy that some syncytia showed TUNEL positivity affecting only a few nuclei or one single nucleus, indicating that the apoptotic process may occur in an asynchronous manner in different nuclei within the same cytoplasm (Figure 1F).

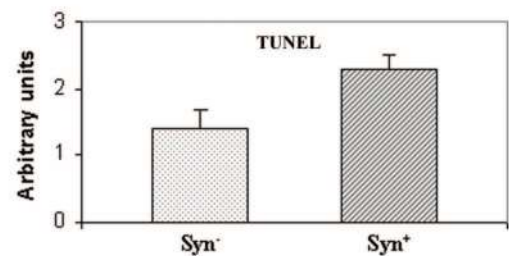
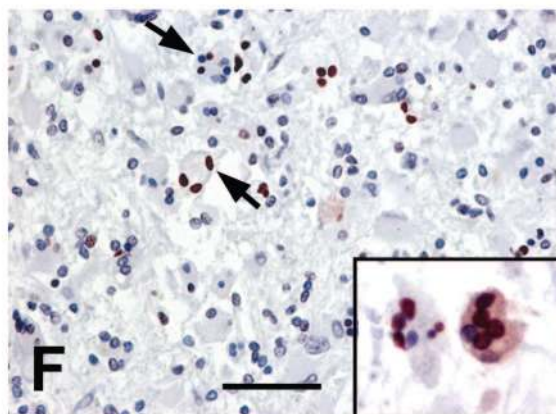
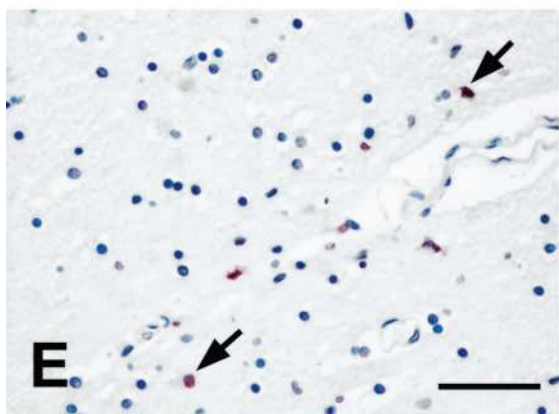
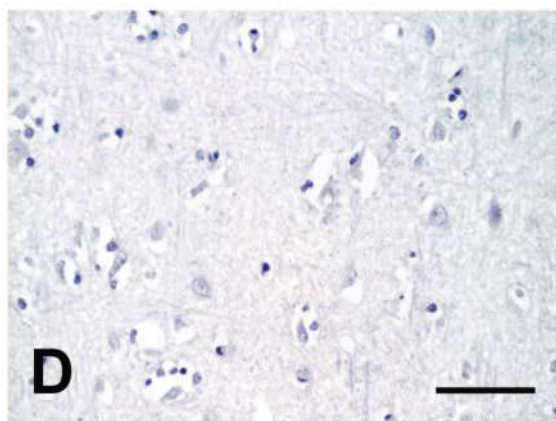
Moreover, it is interesting to note that Syn<sup>+</sup> specimens exhibited a more elevated number of white matter lesions than Syn<sup>-</sup> samples, thus supporting the hypothesis that the presence of syncytia may represent an index of HAE severity (Figure 1H). In fact, the degree of white matter lesions closely correlated with the percentage of syncytia for each specimen ( $R^2 = 0.864$ ,  $P = 0.0072$ ), whereas there was no correlation between number of syncytia and microglial activation.

### Analysis of the Apoptotic Pathway Induced in HIV-Infected Syncytia

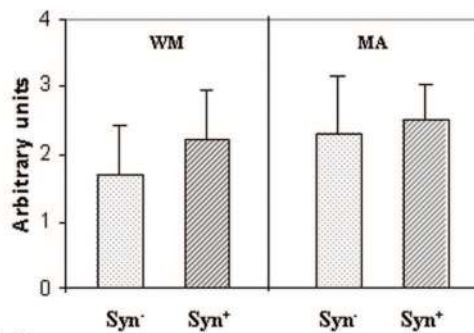
Recent studies underscore the probable importance of p53 for syncytial apoptosis<sup>31–33</sup> as well as for the HIV-1-induced neuropathology.<sup>17</sup> Garden and colleagues<sup>17</sup> de-



**C**



**G**



**H**

scribed the HAE-associated accumulation of p53 protein in cortical neurons and microglia. In lymphocytes from HIV-1-positive patients, we demonstrated the p53 phosphorylation on Ser 15 and on Ser 46 and that these phosphorylations are mediated by the two kinases mTOR and p38 MAPK, respectively.<sup>31,32</sup> Syncytia of HAE patients displayed not only the presence of p38 MAPK,<sup>33</sup> but also mTOR (Figure 2A). The effective activation of mTOR in apoptotic syncytia was confirmed by analyzing the nuclear localization of its downstream target, p70 S6 kinase (Figure 2B). The phosphorylation of p53 on Ser 46 (p53S46P) was observed in  $72.3\% \pm 10.2$  of brain syncytia (Figure 2C). We have previously demonstrated that NF- $\kappa$ B is required for the mTOR-mediated p53 phosphorylation.<sup>33</sup> Accordingly, we detected the activation of NF- $\kappa$ B revealed by the presence of I $\kappa$ B- $\alpha$  phosphorylated on Ser 32 and Ser 36 (I $\kappa$ BS32/36P) in  $42.7\% \pm 25.3$  of syncytia, suggesting that NF- $\kappa$ B is indeed released from I $\kappa$ B-mediated inhibition (Figure 2D). These data confirm the involvement of all these factors also in HAE syncytial apoptosis.

In Figure 2, E and F, we report the brain sections from HIV-negative patients showing no expression of I $\kappa$ B- $\alpha$  phosphorylated on Ser 32 and Ser 36 (Figure 2E) and the detection of TG2 restricted to endothelial cells (Figure 2F, arrowheads), in which the enzyme is constitutively expressed. The specificity of the used staining procedures was confirmed by performing the methodological controls such as the omission of primary antibodies (Figure 2G) and the utilization of nonimmune isotype-matched control antibodies (Figure 2H) on HIV-positive patients.

Next, we determined the expression of one particular p53 target, the BH3-only protein Puma, which has been found to be essential for the death of HIV-1 Env-elicited syncytia.<sup>33</sup> Enhanced Puma expression was consistently observed in HAE brain sections (Figure 3, A and B) suggesting that Puma could be a mediator of p53 signaling involved in the neuropathology. Syncytia exhibited a variable degree of Puma expression in their cytoplasm (Figure 3B); at least  $53.16\% \pm 23.09$  multinucleated cells were judged to contain more Puma than adjacent non-syncytial cells. Further support to the role of Puma in p53-dependent killing of multinucleated cells was obtained by detecting its up-regulation in syncytia showing DNA fragmentation (Figure 3E).

The expression of TG2, another proapoptotic BH3-only protein<sup>35</sup> involved in viral-induced pathologies<sup>36,37</sup> and in the regulation of apoptosis of HIV-infected cells,<sup>38–40</sup> was also analyzed in the same samples (Figure 3, C and D). As expected, high levels of TG2 were found in the cytoplasm of HAE syncytia ( $64.6\% \pm 16.8$  positive) (Ta-

**Table 2.** Histochemical Characteristics of Syncytia in HAE Brain Sections (Number of Patients = 6)

| Characteristics                    | Frequency of stained syncytia ( $\pm$ SD) |
|------------------------------------|---|
| HIV-p24 staining                   | 90.25 $\pm$ 11.09                         |
| I $\kappa$ B- $\alpha$ (Ser32/36P) | 42.7 $\pm$ 25.3                           |
| mTOR                               | 87.77 $\pm$ 14.37                         |
| p53 (Ser46P)                       | 72.3 $\pm$ 10.2                           |
| Puma                               | 53.16 $\pm$ 23.09                         |
| TG2                                | 64.6 $\pm$ 16.8                           |
| TUNEL positivity                   | 48.9 $\pm$ 23                             |

ble 2 and Figure 3, C and D) and, by confocal microscopy studies, we observed its localization exactly in the syncytia showing DNA fragmentation (Figure 3F). TG2 was also detected in endothelial cells, which constitutively express the enzyme (Figure 2F).<sup>41</sup> Interestingly,  $\sim 30\%$  of TUNEL-negative syncytia expressed mTOR and p53S46P (but not Puma or TG2) corroborating a hypothetical sequence of events in which the activation of p53 (as indicated by mTOR induction and p53 phosphorylation) precedes the transactivation of Puma and TG2, both of which act as terminal effectors.

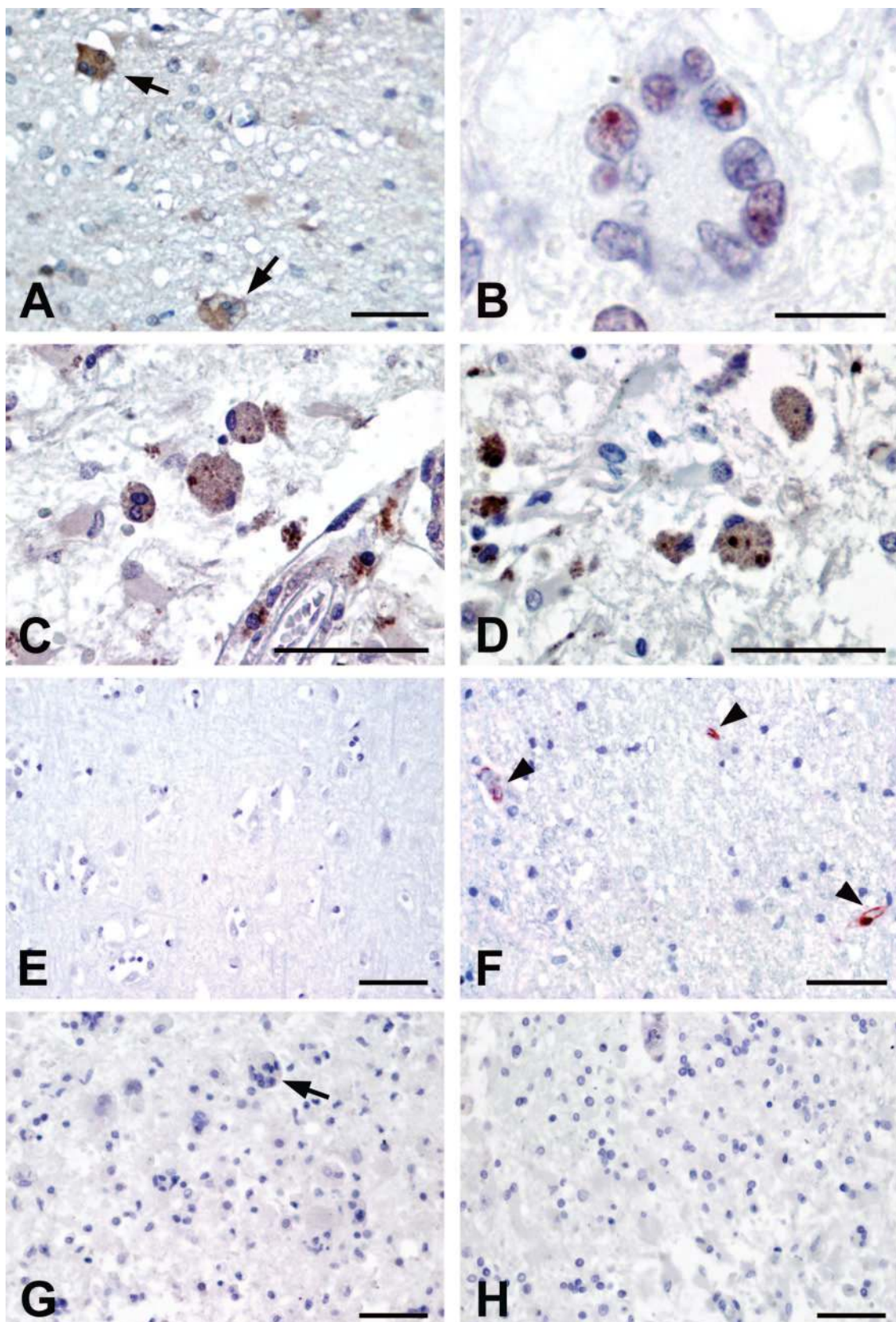
### Analysis of the Apoptotic Pathway Induced in Neurons in HIV-Infected Brains

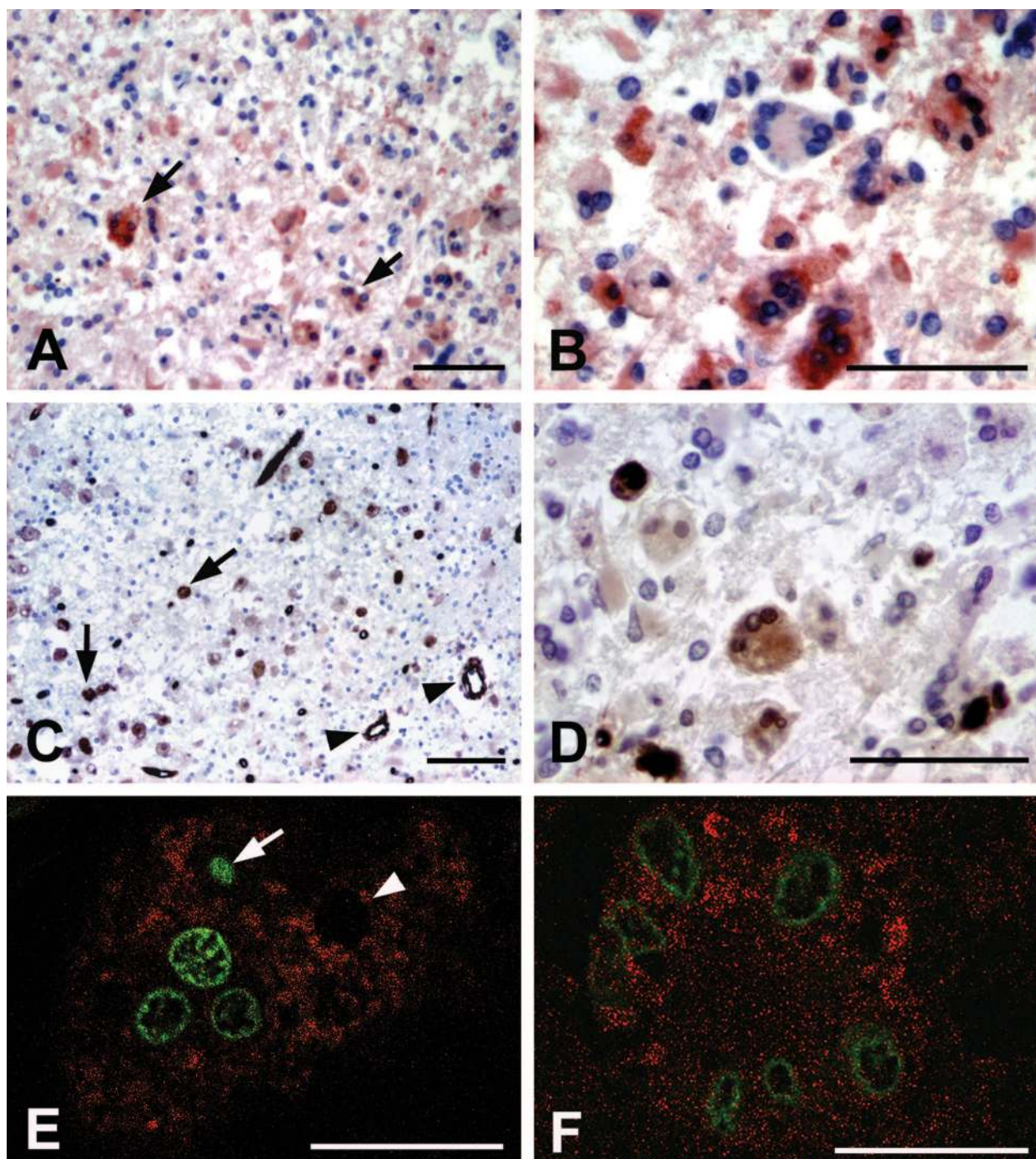
Several studies indicate that HAE is associated with neuronal apoptosis.<sup>8–11</sup> As observed for syncytia, a fraction of neurons manifested the up-regulation of mTOR ( $44.8\% \pm 19.7$ ) and Puma induction ( $56.8\% \pm 7.5$ ) (Figure 4, A and B). Again, the same cells, which expressed Puma, showed extensive DNA fragmentation as detected by TUNEL staining ( $53.4\% \pm 17.8$ ) (Figure 4, C and D). As recently reported, neuronal cells showing Puma-expression are primed toward apoptosis.<sup>42</sup> By contrast, I $\kappa$ B- $\alpha$  phosphorylation and TG2 induction were not observed in the neuronal populations, thus suggesting that neuronal and syncytial cell death follows partially overlapping, yet distinct apoptotic pathways.

### Discussion

The introduction of highly active anti-retroviral therapy has modified the observed disease progression in HIV-induced encephalitis and AIDS-related CNS opportunistic infections. After the introduction of protease inhibitors, a decline in both HIV encephalitis and CNS opportunistic infections was observed.<sup>1,7,24,40</sup> However, with the in-

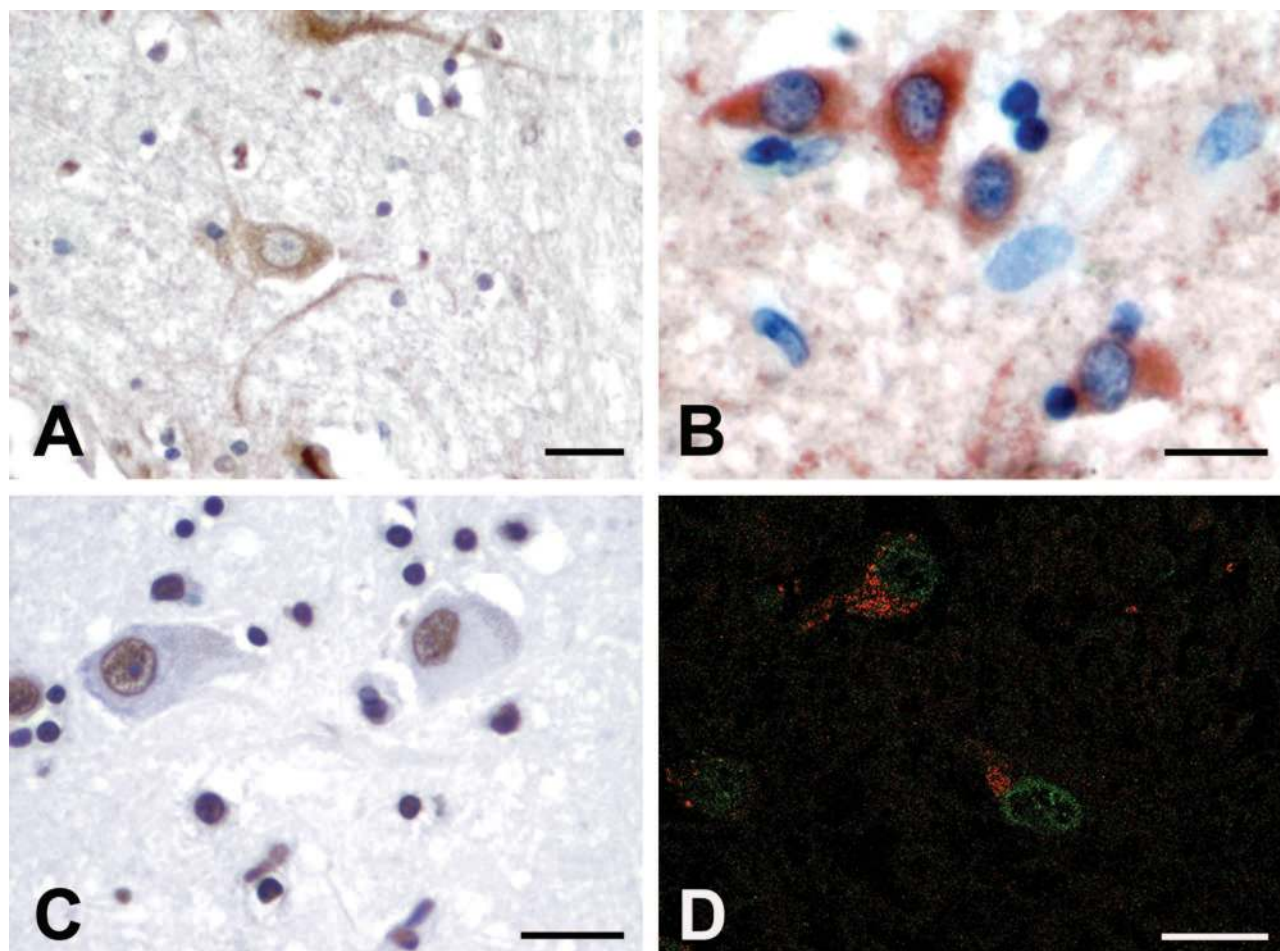
**Figure 1. A–D:** Immunohistochemical analysis of HIV-1 p24 protein expression in frontal cortex sections from patients with HIV-related encephalitis and controls. **A:** Section from a patient with HAE without syncytia. p24 staining is present on cells localized preferentially in perivascular area (**arrows**). **B:** Section from a patient with HAE characterized by the presence of numerous syncytia and an altered tissue architecture. Syncytia (**arrows**) stain for p24 inside the cytoplasm, as visible in the higher magnification **inset**. **C:** Quantification of p24 protein expression on brain section from patients with HIV-related encephalitis with brain syncytia (Syn<sup>+</sup>) and without syncytia (Syn<sup>-</sup>). Statistical analysis underlines a significant difference (\*) of p24 staining between the two categories ( $P = 0.015$ ). **D:** Frontal cortex section from a control subject negative after staining for HIV-p24. **E–H:** TUNEL staining of frontal cortex sections from HIV-related encephalitis patients and controls. **E:** Representative section from a patient with HAE without syncytia. Only a few cells are apoptotic, as indicated by the red nuclear staining (**arrows**). **F:** Staining from a patient with brain syncytia. TUNEL-positive syncytia are visible (**arrows**). Note that in some examples not all of the nuclei of the same syncytium are TUNEL-positive (**inset**). **G:** Quantification of TUNEL-positive cells on brain section from Syn<sup>+</sup> and Syn<sup>-</sup> patients. **H:** Quantification of white matter lesions (WM) and microglia activation (MA) on frontal cortex sections between the two groups of patients: with brain syncytia (Syn<sup>+</sup>) and without this cell type (Syn<sup>-</sup>). Scale bars, 100  $\mu$ m.





**Figure 3.** Immunohistochemistry of syncytia present on frontal cortex sections from patients with HIV-related encephalitis. Analysis of p53-dependent effectors of the apoptotic pathway. **A:** Brain section from a patient, displaying altered tissue architecture, with high expression of Puma protein in syncytia (arrows). Also other histiomaacrophagic cells are labeled. **B:** Higher magnification displays a variable degree of Puma expression in different syncytia. **C:** TG2 labeling. Numerous syncytia are labeled (arrows). Endothelial cells constitutively express TG2 (arrowheads). **D:** Higher magnification displays syncytia exhibiting cytoplasmic expression of TG2. **E:** Confocal microscopy image of a syncytium showing cytoplasmic Puma expression (labeled with Alexa 546, red) and nuclear TUNEL reaction product (fluorescein, green). The syncytium nuclei show different stage of apoptosis; in fact, condensed nucleus (arrow) is present in the same syncytium with TUNEL-negative (arrowhead) and TUNEL-positive nuclei. **F:** Confocal microscopy image of a syncytium stained with anti-TG2 (labeled with Alexa 546, red) and nuclear TUNEL reaction product (fluorescein, green). Scale bars: 100  $\mu$ m (A–D); 25  $\mu$ m (E, F).

**Figure 2.** Immunohistochemical analysis of syncytia present on frontal cortex section from HIV-related encephalitis affected patients and controls. **A:** The section, from a patient with HAE, shows syncytia positively stained for mTOR (arrows). **B:** Syncytium showing nuclear staining for p70 S6 kinase. **C:** Syncytia showing p53 phosphorylated on Ser 46. **D:** Numerous syncytia are labeled for  $\text{I}\kappa\text{B-}\alpha$  phosphorylated on Ser 32/36 that is a marker for NF- $\kappa\text{B}$  activation. **E:** Brain section from control subject submitted to immunostaining for TG2. No labeling is detectable. **F:** Brain section from control subject stained with anti-TG2. The immunoreaction product is exclusively localized on endothelial cells (arrowheads) where TG2 is constitutively expressed. **G:** Frontal cortex section from a patient with HAE submitted to immunostaining by omitting primary antibody and using biotinylated anti-mouse IgG as secondary antibody. No labeling is detectable. The arrow points to a negative syncytium. **H:** Frontal cortex section from a patient with HAE submitted to immunostaining using isotype-matched mouse IgG as negative control. Scale bars: 100  $\mu$ m (A, C–H); 25  $\mu$ m (B).



**Figure 4.** Immunohistochemical analysis and TUNEL staining on neurons from patients with HIV-related encephalitis. **A:** The microphotograph shows neurons stained for mTOR. The brown reaction product is uniformly distributed on the cytoplasm. **B:** Expression of Puma protein on frontal cortex neurons. The cytoplasm of numerous neurons is positively stained with anti-Puma antibody. **C:** Neurons showing DNA fragmentation detectable by brown precipitate in the nucleus. **D:** Confocal microscopy image showing Puma expression (labeled with Alexa 546, red) and TUNEL reactivity (fluorescein, green) in neurons. Scale bars, 50  $\mu\text{m}$ .

creasing resistance of HIV strains to anti-retrovirals, there has been a rebound in the frequency of HIV encephalitis and HIV leukoencephalopathy. The brain is considered as a potential HIV reservoir; in fact the virus can persist even after long periods of treatment with highly active anti-retroviral therapy.<sup>1,7,24,40,43</sup>

The mechanistic dissection of HAE should be envisaged to elaborate novel therapeutic strategies. To this aim, we have analyzed HIV-induced apoptotic pathway(s) in brain sections from HAE-affected patients. We described a substantial difference between HAE cases showing the presence or the absence of syncytia. On the basis of our results, syncytia can be considered as an index of HAE severity because their presence correlates with an elevated number of HIV-1-infected cells (as detected by p24 staining). In addition, a pronounced apoptosis (as detected by TUNEL staining) and histopathological brain alterations (white matter lesions and microglia activation) are also detected in the same patients.

As far as the cell death mechanisms involved in the demise of brain cells are concerned, we revealed the expression on brain syncytia of the tumor suppressor

transcription factor p53 phosphorylated on Ser 46. p53 phosphorylation is known to increase the transactivating function of p53<sup>44</sup> and thus may contribute to apoptosis induction.<sup>45,46</sup> We previously showed that phosphorylated p53 plays a key role in the death of syncytia elicited by HIV infection *in vitro*.<sup>31</sup> In keeping with these findings, an increased p53 protein expression in its proapoptotic form has been revealed in syncytia of HAE patients, indicating that p53-dependent death signaling is required for HIV-induced degeneration of multinucleated cells. We previously reported the involvement of mTOR in the phosphorylation of p53 in lymphoid tissues of HIV-infected patients.<sup>31,32</sup> Here, we show that mTOR is not only highly activated in brain syncytia of HIV-infected patients, thus suggesting its general participation in the lethal p53-driven signaling cascade involved in HAE. We detected the presence of the BH3-only protein Puma in brain syncytia, especially in TUNEL-positive syncytia.

Another proapoptotic protein, TG2, was also found in the cytoplasm of syncytia, especially at an advanced stage of the process, when syncytia were TUNEL-positive. TG2 has been shown to play an important role in neurodegenerative pathologies, for instance in Hunting-



ton's disease.<sup>47</sup> Recently, TG2 has been shown to act as a multifunctional BH3-only protein at the mitochondrial level.<sup>35</sup>

Altogether, the results obtained in this study are compatible with the notion that the formation and death of syncytia correlates with or contributes to the neuropathology of HAE. Once formed, presumably as the result of a fusion process involving at least one HIV-infected (p24<sup>+</sup>) cell, syncytia activate a lethal signal transduction cascade whose early events (mTOR up-regulation and I $\kappa$ B- $\alpha$  phosphorylation) manifest before cells become TUNEL-positive, whereas late events (Puma and TG2 induction) coincide with cellular demise.

Some of the factors involved in syncytial cell death are overexpressed also in neurons of HAE patients. In fact, we detected the neuronal up-regulation of mTOR and Puma, thus suggesting their participation to the neuronal death pathway. In keeping with this assumption, the overexpression of Puma during neuronal cell death has been recently described.<sup>42</sup>

However, dying cortical neurons found in HAE patients were TG2-negative, suggesting that neuronal and syncytial death obeys different rules. In accord with this speculation, neurons also lacked signs of NF- $\kappa$ B activation, which were detected in HAE syncytia. These findings suggest that neuronal and syncytial cell death follows partially overlapping, yet distinct apoptotic pathways. This knowledge may lay the ground for future studies and intervention on HAE-related cell death.

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